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ViraPower™ HiPerform™ T-Rex™ Gateway® Expression System

Gateway®-adapted lentiviral systems for regulated, high-level expression in dividing and non-dividing mammalian cells

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User Manual

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Kit Contents and Storage

System Components

The ViraPower™ HiPerform™ T-REx™ Gateway® Expression System includes the ViraPower™ HiPerform™ T-REx™ Gateway® Vector Kit, ViraPower™ Lentiviral Support Kit, 293FT Cell Line, LR Clonase™ II Enzyme Mix, One Shot® Stbl3™ Chemically Competent *E. coli*, Geneticin® and Blasticidin selection agents, and pENTR Gus positive control plasmid. For a detailed description of the contents of each component, see pages v–vi.

Shipping/Storage

The ViraPower™ HiPerform™ T-REx™ Gateway® Expression System components are shipped as described below. Upon receipt, store each component as detailed below.

Component	Shipping	Storage
ViraPower™ HiPerform™ T-REx™ Gateway® Vector Kit: • Vectors • Tetracycline	Dry ice	 –20°C –20°C (**protected from light**)
ViraPower™ Lentiviral Support Kit: • ViraPower™ Packaging Mix • Lipofectamine™ 2000	Blue ice	 –20°C 4°C **(do not freeze)**
293FT Cells	Dry ice	Liquid nitrogen
pENTR™ Gus Positive Control	Blue ice	–20°C
One Shot® Stbl3™ Chemically Competent *E. coli*	Dry ice	–80°C
Gateway® LR Clonase™ II Enzyme Mix	Dry ice	–20°C
Geneticin®, liquid	Blue ice	4°C or –20°C
Blasticidin	Blue ice	–20°C

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ViraPower™ HiPerform™ T-REx™ Gateway® Vector Kit

The following reagents are included with the ViraPower™ HiPerform™ T-REx™ Gateway® Vector Kit. Store the vectors at –20°C. Store the tetracycline at –20°C, protected from light.

Reagent	Composition	Amount
pLenti6.3/TO/V5-DEST	40 µL of vector at 150 ng/µL in TE Buffer, pH 8.0*	6 µg
pLenti3.3/TR	40 µL of vector at 0.5 µg/µL in TE Buffer, pH 8.0	20 µg
pLenti6.3/TO/V5-GW/*lac*Z	20 µL of vector at 0.5 µg/µL in TE Buffer, pH 8.0	10 µg
Tetracycline	10 mg/mL in water	1 mL

***TE Buffer, pH8.0:** 10 mM Tris-HCl, 1 mM EDTA, pH 8.0

ViraPower™ Lentiviral Support Kit Contents

The ViraPower™ Lentiviral Support Kit includes the following vectors and reagents. Store the ViraPower™ Packaging Mix at –20°C.
Store Lipofectamine™ 2000 at 4°C (do not freeze).

Reagent	Composition	Amount
ViraPower™ Packaging Mix	Contains a mixture of the pLP1, pLP2, and pLP/VSVG plasmids in TE Buffer, pH 8.0	195 µL
Lipofectamine™ 2000	Proprietary	0.75 mL

293FT Cells

Each ViraPower™ HiPerform™ T-REx™ Gateway® Expression System includes the 293FT producer cell line. The 293FT Cell Line is supplied as one vial containing 3×10^6 frozen cells in 1 mL of Freezing Medium. **Upon receipt, store in liquid nitrogen.**

For instructions to thaw, culture, and maintain the 293FT Cell Line, see the 293FT Cell Line manual, included in the ViraPower™ HiPerform™ T-REx™ Gateway® Expression System. The 293FT Cell Line manual is also available for downloading at www.invitrogen.com, or by contacting Technical Support (see page 60).

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One Shot® Stbl3™ Chemically Competent *E. coli*

The following reagents are included with the One Shot® Stbl3™ Chemically Competent *E. coli* kit. Transformation efficiency is ≥ 1×10^8 cfu/µg plasmid DNA. **Store at –80°C.**

Reagent	Composition	Amount
S.O.C. Medium	2% Tryptone 0.5% Yeast Extract 10 mM NaCl 2.5 mM KCl 10 mM $MgCl_2$ 10 mM $MgSO_4$ 20 mM glucose	6 mL
Stbl3™ Cells	--	21 × 50 µL
pUC19 Control DNA	10 pg/µL in 5 mM Tris-HCl, 0.5 mM EDTA, pH 8.0	50 µL

Genotype of Stbl3™ Cells

F$^-$ *mcr*B *mrr hsd*S20(r_B^-, m_B^-) *rec*A13 *sup*E44 *ara*-14 *gal*K2 *lac*Y1 *pro*A2 *rps*L20(Str^R) *xyl*-5 λ$^-$ *leu mtl*-1

Note: This strain is *end*A1+

Gateway® LR Clonase™ II Plus Enzyme Mix

The following reagents are included with the Gateway® LR Clonase™ II Plus Enzyme Mix. **Store the Gateway® LR Clonase™ II Plus Enzyme Mix components at –20°C for up to 6 months.** For long-term storage, store at –80°C.

Reagent	Composition	Amount
Gateway® LR Clonase™ II Enzyme Mix	Proprietary	40 µL
Proteinase K Solution	2 µg/mL in: 10 mM Tris-HCl, pH 7.5 20 mM $CaCl_2$ 50% glycerol	40 µL

Blasticidin

The ViraPower™ HiPerform™ T-REx™ Gateway® Expression System includes Blasticidin for selection of stable cell lines expressing your gene of interest from pLenti6.3/TO/V5-DEST. Blasticidin is supplied as 50 mg of powder. **Store at –20°C.**

Geneticin®

The ViraPower™ HiPerform™ T-REx™ Gateway® Expression System includes Geneticin® for selection of stable cell lines expressing the Tet repressor from pLenti3.3/TR. Geneticin® is supplied as 20 mL of 50 mg/mL solution in distilled water. **Store at 4°C.**

Introduction

Description of the System

ViraPower™ Lentiviral Technology

ViraPower™ Lentiviral Technology facilitates highly efficient, *in vitro* or *in vivo* delivery of a target gene or RNA to dividing and non-dividing mammalian cells using a replication-incompetent lentivirus. Based on the lentikat™ system developed by Cell Genesys (Dull *et al.*, 1998), the ViraPower™ Lentiviral Technology possesses features which enhance its biosafety and allow high-level expression in a wider range of cell types than traditional retroviral systems. For more information about the biosafety features of the System, see pages 4–5.

How Lentivirus Works

After the lentivirus enters the target cell, the viral RNA is reverse-transcribed, actively imported into the nucleus (Lewis & Emerman, 1994; Naldini, 1999), and stably integrated into the host genome (Buchschacher & Wong-Staal, 2000; Luciw, 1996). After the lentiviral construct integrates into the genome, you may assay for transient expression of your recombinant protein or use antibiotic selection to generate a stable cell line for long-term expression studies.

Components of the ViraPower™ HiPerform™ T-REx™ Gateway® Expression System

The ViraPower™ HiPerform™ T-REx™ Gateway® Expression System combines Invitrogen's ViraPower™ HiPerform™ Lentiviral and T-REx™ technologies to facilitate lentiviral-based, regulated, high-level expression of a target gene in dividing or non-dividing mammalian cells. The System includes:

- The pLenti6.3/TO/V5-DEST destination vector into which the gene of interest is cloned. The tetracycline-regulated, hybrid CMV/TO promoter controls the expression of the cloned gene. This destination vector also contains the polypurine tract from HIV (cPPT) for increased viral titer (Park et al., 2001), the Woodchuck Posttranscriptional Regulatory Element (WPRE) for increased transgene expression (Zufferey et al., 1999), elements that allow packaging of the expression construct into virions, and the Blasticidin resistance marker for selecting stably transduced cell lines.
- The pLenti3.3/TR repressor plasmid that constitutively expresses high levels of the tetracycline (Tet) repressor under the control of a CMV promoter. This plasmid also contains elements that allow viral packaging and the Neomycin resistance marker for selecting stably transduced cell lines.
- The ViraPower™ Packaging Mix that contains an optimized mixture of the three packaging plasmids, pLP1, pLP2, and pLP/VSVG. These plasmids supply the helper functions as well as structural and replication proteins *in trans* required to produce the lentivirus. For more information about the packaging plasmids, see the **Appendix**, pages 42-57.
- The 293FT producer cell line that stably expresses the SV40 large T antigen under the control of the human CMV promoter for optimal virus production. For more information about the 293FT Cell Line, refer to the 293FT Cell Line manual.
- Lipofectamine™ 2000 reagent for high-efficiency transfection of 293FT producer cell line.

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Advantages of the ViraPower™ HiPerform™ T-REx™ Gateway® Expression System

- Generates replication-incompetent lentivirus that transduces dividing and non-dividing mammalian cells, thus broadening the potential applications beyond those of other traditional retroviral systems (Naldini, 1998).
- Efficiently delivers the gene of interest and the Tet repressor to mammalian cells in culture or *in vivo.* Expression of the target gene is regulated by tetracycline.
- Provides stable, long-term, tetracycline-regulated expression of a target gene beyond that offered by traditional adenoviral-based systems.
- Produces a pseudotyped virus with a broadened host range (Yee, 1999).
- Allows enhanced protein expression, up to 4-fold or greater, compared to traditional lentiviral expression systems.
- Provides Gateway®-adapted expression vector for easy recombination-based cloning of any gene of interest.
- Includes multiple features designed to enhance the biosafety of the system.

T-REx™ Technology

T-REx™ Technology facilitates tetracycline-regulated expression of a gene of interest in mammalian cells through the use of regulatory elements from the *E. coli* Tn*10*-encoded tetracycline (Tet) resistance operon (Hillen & Berens, 1994; Hillen *et al.*, 1983). Tetracycline regulation in the T-REx™ System is based on the binding of tetracycline to the Tet repressor and derepression of the promoter controlling expression of the gene of interest (Yao *et al.*, 1998).

T-REx™ Technology uses an inducible expression construct containing the gene of interest under a hybrid promoter consisting of the human cytomegalovirus (CMV) promoter and two tetracycline operator 2 ($TetO_2$) sites and a regulatory expression construct that facilitates high-level, constitutive expression of the Tet repressor (TetR).

When the inducible expression construct and the regulatory expression construct are present in the same mammalian cell, expression of the gene of interest is repressed in the absence of tetracycline and induced in its presence (Yao *et al.*, 1998).

HiPerform™ Technology

The lentiviral expression vectors in ViraPower™ HiPerform™ T-REx™ Gateway® Expression System contain two genetic elements (WPRE and cPPT) that enhance viral titer and expression in certain cell types. The **WPRE** (Woodchuck Posttranscriptional Regulatory Element) from the woodchuck hepatitis virus, is placed directly downstream of the gene of interest, thereby increasing the nuclear export of the transcript and enhancing transgene expression (Mastroyiannopoulos *et al.*, 2005; Zufferey *et al.*, 1998). The **cPPT** (Polypurine Tract) from the HIV-1 integrase gene, increases the copy number of lentivirus integrating into the host genome (Park, 2001) and allows for a two-fold increase in viral titer. WPRE and cPPT together produce at least a four-fold increase in protein expression in most cell types, compared to other vectors that do not contain these elements.

Continued on next page

Description of the System, Continued

The Gateway® Technology

Gateway® Technology is a universal cloning method that takes advantage of the site-specific recombination properties of bacteriophage lambda (Landy, 1989) to provide a rapid and highly efficient way to move your DNA sequence of interest into multiple vector systems. To generate an expression contruct containing your gene of interest, simply:

1. Clone your gene of interest into a Gateway® entry vector of choice to create an entry clone.

   Note: The Gateway® entry vector is not included in the ViraPower™ HiPerform™ T-REx™ Gateway® Expression System.

2. Generate an expression clone by performing an LR recombination reaction between the entry clone and the pLenti6.3/TO/V5-DEST destination vector.

For detailed information about the Gateway® Technology, refer to the Gateway® Technology with Clonase™ II manual which is available at www.invitrogen.com or by contacting Technical Support (see page 62).

Purpose of this Manual

This manual provides an overview of the ViraPower™ HiPerform™ T-REx™ Gateway® Expression System and provides instructions and guidelines to:

1. Co-transfect the pLenti-based expression vector and the ViraPower™ Packaging Mix into the 293FT Cell Line to produce a lentiviral stock.
2. Titer the lentiviral stock.
3. Use the lentiviral stock to transduce your mammalian cell line of choice.
4. Assay for "transient" expression of your recombinant protein, or
5. Generate a stably transduced cell line, if desired.

For details and instructions to generate your expression vector, refer to the ViraPower™ HiPerform™ T-REx™ Gateway® Vector Kit manual. For instructions to culture and maintain the 293FT producer cell line, refer to the 293FT Cell Line manual. These manuals are supplied with the ViraPower™ HiPerform™ T-REx™ Gateway® Expression System, and are also available for downloading from our website at www.invitrogen.com or by contacting Technical Support (page 62).

Biosafety Features of the System

Introduction

The ViraPower™ HiPerform™ T-REx™ Gateway® Expression System is based on lentiviral vectors developed by Dull *et al.*, 1998 includes a significant number of safety features designed to enhance its biosafety and to minimize its relation to the wild-type, human HIV-1 virus.

Biosafety Features of the ViraPower™ HiPerform™ Lentiviral System

- The pLenti expression vector contains a deletion in the 3′ LTR (ΔU3) that does not affect generation of the viral genome in the producer cell line, but results in "self-inactivation" of the lentivirus after transduction of the target cell (Yee *et al.*, 1987; Yu *et al.*, 1986; Zufferey *et al.*, 1998). Once integrated into the transduced target cell, the lentiviral genome is no longer capable of producing packageable viral genome.
- The number of genes from HIV-1 that are used in the system has been reduced to three (i.e., *gag*, *pol*, and *rev*).
- The VSV-G gene from Vesicular Stomatitis Virus is used in place of the HIV-1 envelope (Burns *et al.*, 1993; Emi *et al.*, 1991; Yee *et al.*, 1994).
- Genes encoding the structural and other components required for packaging the viral genome are separated onto four plasmids. All four plasmids have been engineered not to contain any regions of homology with each other to prevent undesirable recombination events that could lead to the generation of a replication-competent virus (Dull *et al.*, 1998).
- Although the three packaging plasmids allow expression *in trans* of proteins required to produce viral progeny (e.g., gal, pol, rev, env) in the 293FT producer cell line, none of them contain LTRs or the Ψ packaging sequence. This means that none of the HIV-1 structural genes are actually present in the packaged viral genome, and thus, are never expressed in the transduced target cell. No new replication-competent virus can be produced.
- The lentiviral particles produced in this system are replication-incompetent and only carry the gene of interest. No other viral species are produced.
- Expression of the *gag* and *pol* genes from pLP1 has been rendered Rev-dependent by virtue of the HIV-1 RRE in the gag/pol mRNA transcript. Addition of the RRE prevents gag and pol expression in the absence of Rev (Dull *et al.*, 1998).
- A constitutive promoter (RSV promoter) has been placed upstream of the 5′ LTR in the pLenti expression vector to offset the requirement for Tat in the efficient production of viral RNA (Dull *et al.*, 1998).

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Biosafety Features of the System, Continued

Biosafety Level 2

Despite the inclusion of the safety features discussed on the previous page, the lentivirus produced with this System can still pose some biohazardous risk, because it can transduce primary human cells.

We highly recommend that you treat lentiviral stocks generated using this System as Biosafety Level 2 (BL-2) organisms and strictly follow all published BL-2 guidelines with proper waste decontamination. Furthermore, exercise extra caution when creating lentivirus carrying potential harmful or toxic genes (e.g., activated oncogenes).

For more information about the BL-2 guidelines and lentivirus handling, refer to the document, *Biosafety in Microbiological and Biomedical Laboratories*, 5th Edition, published by the Centers for Disease Control (CDC). You can download this document from the following address:

www.cdc.gov/od/ohs/biosfty/bmbl5/bmbl5toc.htm

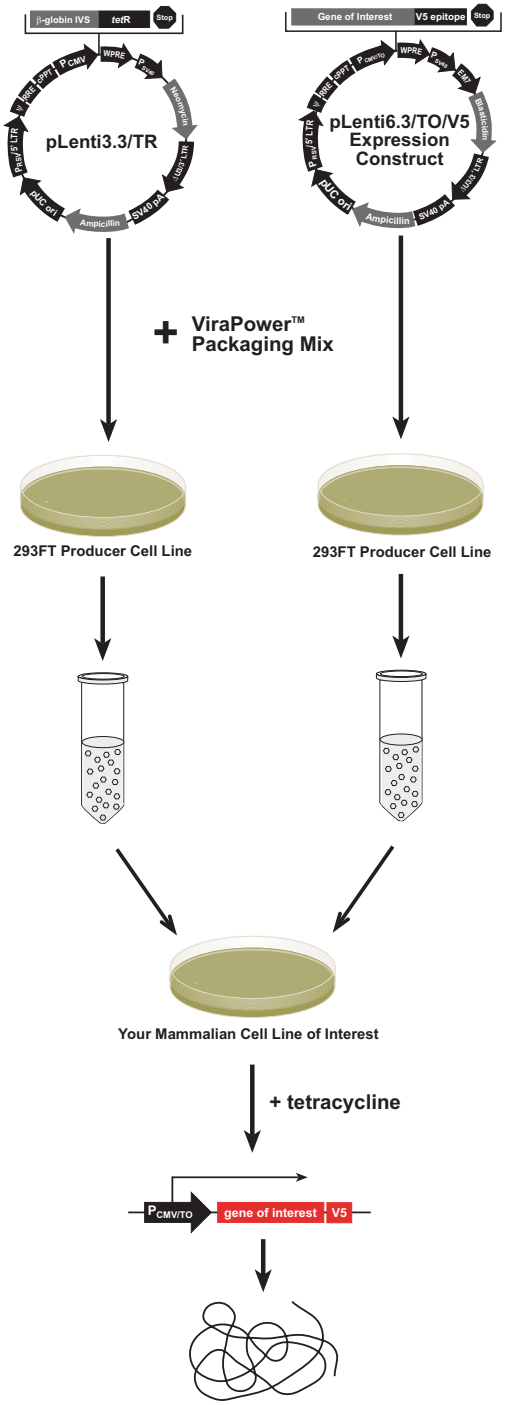
Important

Handle all lentiviruses in compliance with established institutional guidelines. Since safety requirements for use and handling of lentiviruses may vary at individual institutions, we recommend consulting the health and safety guidelines and/or officers at your institution prior to use of the ViraPower™ HiPerform™ T-REx™ Gateway® Expression System.

Experiment Outline

Flow Chart

The diagram below describes the general steps required to express your gene of interest using the ViraPower™ HiPerform™ Lentiviral Expression System. Refer to the ViraPower™ HiPerform™ T-REx™ Gateway® Vector Kit (part no. A11226) manual for instructions to generate your pLenti expression construct.



1. Generate the pLenti6.3/TO/V5 expression construct containing your gene of interest.

2. Cotransfect the 293FT producer cell line with pLenti3.3/TR or your pLenti6.3/TO/V5 expression construct and the optimized Packaging Mix.

3. Harvest viral supernatant and determine the titer.

4a. Add the Lenti3.3/TR viral supernatant to your mammalian cell line and use Neomycin selection to generate a ViraPower™ T-REx™ cell line. Add the Lenti6.3/TO/V5 viral supernatant to the ViraPower™ T-REx™ cells, and use Blasticidin to select for stably transduced cells, if desired.

OR

4b. Co-transduce your mammalian cell line with the Lenti3.3/TR and Lenti6.3/TO/V5 viral supernatants. Select for stably transduced cells, if desired.

5. Add tetracycline. Assay for recombinant protein of interest.

Methods

General Information

Introduction

The ViraPower™ HiPerform™ T-REx™ Gateway® Expression System is designed to help you create a lentivirus to deliver and express a gene of interest in mammalian cells. Although the system has been designed to help you express your recombinant protein of interest in the simplest, most direct fashion, use of the system is geared towards those users who are familiar with the principles of retrovirus biology and retroviral vectors. We highly recommend that users possess a working knowledge of virus production and tissue culture techniques. For more information about these topics, refer to the following published reviews:

- Retrovirus biology and the retroviral replication cycle: see Buchschacher and Wong-Staal (2000) and Luciw (1996) .
- Retroviral and lentiviral vectors: see Naldini (1998), Naldini (1999), Yee (1999) and Pandya et al., 2001 (Naldini, 1998; Naldini, 1999; Pandya *et al.*, 2001; Yee, 1999).

Positive Control

We recommend including a positive control vector in your co-transfection experiment to generate a control lentiviral stock that you can use to optimize expression conditions in your mammalian cell line of interest.

- The ViraPower™ HiPerform™ T-REx™ Gateway® Vector Kit includes the positive control vector pLenti6.3/TO/V5-GW/*lac*Z for use as an expression control.
- A control lentiviral expression vector containing Emerald Green Fluorescent Protein (EmGFP) for fluorescent detection (pLenti6.3/V5-GW/EmGFP) is available separately from Invitrogen (page 60). This control vector expresses EmGFP constitutively, and is not inducible.

Lipofectamine™ 2000

The Lipofectamine™ 2000 reagent supplied with the kit (Ciccarone *et al.*, 1999) is a proprietary, cationic lipid-based formulation suitable for the transfection of nucleic acids into eukaryotic cells. Using Lipofectamine™ 2000 to transfect 293FT cells offers the following advantages:

- Provides the highest transfection efficiency in 293FT cells.
- You can add the DNA-Lipofectamine™ 2000 complexes directly to cells in culture medium in the presence of serum.
- You do not have to remove the complexes or change or add medium following transfection; however, you may remove the complexes 4–6 hours after transfection without loss of activity.

Note: Lipofectamine™ 2000 is available separately from Invitrogen or as part of the ViraPower™ HiPerform™ Lentiviral Support Kits (see page 60).

Opti-MEM® I

To facilitate optimal formation of DNA-Lipofectamine™ 2000 complexes, we recommend using Opti-MEM® I Reduced Serum Medium available from Invitrogen (see page 60).

Generating pLenti Expression Construct

Introduction

To generate a pLenti expression construct containing your gene of interest, refer to the ViraPower™ HiPerform™ T-REx™ Gateway® Vector Kit manual (part no. A11226) for instructions. Once you have created your expression construct, isolate plasmid DNA for transfection.

Note: It is important that you verify that your lentiviral plasmid has not undergone aberrant recombination by performing an appropriate restriction enzyme digest. See the vector kit manual for details.

Guidelines for Isolating DNA

Plasmid DNA for transfection into eukaryotic cells must be very clean and free from contamination with phenol and sodium chloride. Contaminants may kill the cells, and salt will interfere with lipid complexing, decreasing transfection efficiency.

When isolating plasmid DNA from *E. coli* strains (such as Stbl3™) that are wild type for endonuclease 1 (*end*A1+) with commercially available kits, ensure that the Lysis or Resuspension Buffer contains 10 mM EDTA. EDTA will inactivate the endonuclease and avoid DNA nicking and vector degradation. Alternatively, follow the instructions included the plasmid purification kits for *end*A1+ *E. coli* strains.

Resuspend the purified plasmid DNA in sterile water or TE Buffer, pH 8.0 to a final concentration ranging from 0.1–3.0 µg/mL. You will need 3 µg of the expression plasmid for each transfection.

Important

Do not use mini-prep plasmid DNA for lentivirus production. We recommend preparing lentiviral plasmid DNA using the PureLink™ HiPure Plasmid MidiPrep kit which contains 10 mM EDTA in the Resuspension Buffer (see page 60 for ordering information).

Continued on next page

Producing Lentivirus in 293FT Cells

Introduction

Before you can create a stably transduced cell line expressing your gene of interest, you need to produce a lentiviral stock (containing the packaged pLenti expression construct) by co-transfecting the optimized packaging plasmid mix and your pLenti expression construct into the 293FT Cell Line. This section provides protocols and instructions to generate a lentiviral stock.

Lentiviral Stocks

To use the ViraPower™ HiPerform™ T-REx™ Gateway® Expression System for regulated expression of your gene of interest, you need to generate lentiviral stocks of the following expression constructs:

- Your pLenti6.3/TO/V5-DEST expression construct containing the gene of interest
- The pLenti3.3/TR construct expressing the Tet repressor (see below for more information)

We also recommend generating a lentiviral stock with the pLenti6.3/TO/V5-GW/*lac*Z control construct for use as a positive control for lentivirus production and expression, if desired. For more information, see the next page.

pLenti3.3/TR

The pLenti3.3/TR plasmid contains the *TetR* gene and the Neomycin resistance marker to allow stable expression of the Tet repressor in any mammalian cell line. To use pLenti3.3/TR:

1. Co-transfect the vector and the ViraPower™ Packaging Mix into 293FT cells to generate a lentiviral stock.
2. Transfect the Lenti3.3/TR lentiviral construct into the mammalian cell line of choice.
3. Use Neomycin selection to generate a stable "ViraPower™ T-REx™" cell line expressing the Tet repressor. The ViraPower™ T-REx™ cell line becomes the host for your Lenti6.3/TO/V5 lentiviral construct.

For the map and features of pLenti3.3/TR, see the **Appendix**, page 50. For the recommended transfection procedures, see **Recommended Procedure**, page 11.

Positive Control

The pLenti6.3/TO/V5-GW/*lac*Z plasmid is supplied with the ViraPower™ HiPerform™ T-REx™ Gateway® Expression System as a control for lentivirus production and expression. We recommend including the positive control vector in your co-transfection experiment to generate a control lentiviral stock. Transducing the control lentivirus into a ViraPower™ T-REx™ cell line allows tetracycline-regulated expression of a C-terminal, V5 epitope-tagged β-galactosidase fusion protein that you can easily detect by western blot or functional assay. For details about the features of the vector, refer to the ViraPower™ HiPerform™ T-REx™ Gateway® Vector Kit manual.

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ViraPower™ Packaging Mix

The pLP1, pLP2, pLP/VSVG plasmids are provided in an optimized mixture to facilitate viral packaging of your pLenti expression vector following co-transfection into 293FT producer cells. The amount of the packaging mix (195 µg at 1 µg/µL) and Lipofectamine™ 2000 transfection reagent (0.75 mL) supplied with the ViraPower™ HiPerform™ T-REx™ Gateway® Expression System is sufficient to perform 20 co-transfections in 10 cm plates.

Note: ViraPower™ Packaging Mix is available separately from Invitrogen or as part of the ViraPower™ Lentiviral Support Kits (page 60).

293FT Cell Line

The human 293FT Cell Line is supplied with the ViraPower™ HiPerform™ T-REx™ Gateway® Expression System to facilitate optimal lentivirus production (Naldini *et al.*, 1996). The 293FT Cell Line, a derivative of the 293F Cell Line, stably and constitutively expresses the SV40 large T antigen from pCMVSPORT6TAg.neo and must be maintained in medium containing Geneticin® (page 60).

For more information about pCMVSPORT6TAg.neo and how to culture and maintain 293FT cells, refer to the 293FT Cell Line manual. This manual, supplied with the ViraPower™ HiPerform™ T-REx™ Gateway® Expression System, is also available by downloading from www.invitrogen.com or by contacting Technical Support (see page 60).

Note: The 293FT Cell Line is also available separately from Invitrogen (page 60).

Guidelines for 293FT Culture

The health of your 293FT cells at the time of transfection has a critical effect on the success of lentivirus production. Use of "unhealthy" cells will negatively affect the transfection efficiency, resulting in production of a low titer lentiviral stock. For optimal lentivirus production (i.e., producing lentiviral stocks with the expected titers), follow the guidelines below to culture 293FT cells before use in transfection:

- Ensure that cells are healthy and greater than 90% viable.
- Subculture and maintain cells in complete medium containing 0.1 mM MEM Non-Essential Amino Acids, 4 mM L-Glutamine, 1 mM sodium pyruvate, 500 µg/mL Geneticin® and 10% fetal bovine serum (FBS) that is not heat-inactivated (page 60).
- Do not allow cells to overgrow before passaging.
- Use cells that have been passaged 3–4 times after the most recent thaw.
- Use cells that have been subcultured for less than 16 passages.

Note

Development work with this kit utilized 293FT cells and 500 µg/mL Geneticin®; however, because different transfected cells may exhibit different Geneticin® sensitivity, we recommend that you conduct a kill curve study to establish the ideal concentration of Geneticin® for using with your cells. See **Determining Geneticin® Sensitivity** in the **Appendix**, page 42, for a kill curve study protocol.

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Recommended Transfection Conditions

We produce lentiviral stocks in 293FT cells using the following **optimized** transfection conditions in the table below. The amount of lentivirus produced using these recommended conditions (10 mL of virus at a titer of at least 1×10^5 transducing units (TU)/mL) is generally sufficient to transduce at least 1×10^6 cells at a multiplicity of infection (MOI) = 1.

For example, you can transduce 10 wells of cells plated at 1×10^5 cells/well in 6-well plates using 1 mL of a 1×10^5 TU/mL virus stock per well to achieve an MOI of 1.

Condition	Quantity
Tissue culture plate size	10 cm (one per lentiviral construct)
Number of 293FT cells to transfect	6×10^6 cells (see **Guidelines for 293FT Culture**, previous page to prepare cells for transfection)
Amount of ViraPower™ Packaging Mix	9 µg (9 µL of 1 µg/µL stock)
Amount of pLenti plasmid (pLenti6.3/TO/V5-DEST expression construct or pLenti3.3/TR repressor plasmid)	3 µg
Amount of Lipofectamine™ 2000	36 µL

Note: You may produce lentiviral stocks using other tissue culture formats provided that you optimize conditions to obtain the expected titers.

Recommended Procedure

If you are producing lentivirus for the first time using the ViraPower™ HiPerform™ T-REx™ Gateway® Expression System and 293FT cells, perform the **Forward Transfection** procedure on page 13. This procedure requires plating the 293FT cells the day before transfection to obtain cells that are 90–95% confluent.

Note: In previous ViraPower™ manuals, this protocol was referred to as the Alternate Transfection Method.

If you are an experienced lentivirus user and are familiar with the growth characteristics of 293FT cells, you may choose to perform the **Reverse Transfection** procedure on page 15. In this procedure, 293FT cells are added directly to media containing the DNA-Lipofectamine™ 2000 complexes.

Continued on next page

Materials Needed

Materials required, but not supplied with the kit:

- pLenti expression vector containing your gene of interest (0.1–3.0 µg/µL in sterile water or TE, pH 8.0)
- 293FT cells cultured in the appropriate medium (i.e., D-MEM containing 10% FBS, 4 mM L-Glutamine, 1 mM MEM sodium pyruvate, 0.1 mM MEM Non-Essential Amino Acids, and 1% penicillin-streptomycin, and 500 µg/mL Geneticin®)

  Note: MEM Sodium Pyruvate provides an extra energy source for the cells and is available from Invitrogen as a 100 mM stock solution (page 60).
- Opti-MEM® I Reduced Serum Medium (pre-warmed to 37°C, page 60)
- Fetal bovine serum (FBS, page 60)
- Complete growth medium **without antibiotics** (i.e., D-MEM containing 10% FBS, 4 mM L-Glutamine, 0.1 mM MEM Non-Essential Amino Acids, and 1 mM MEM sodium pyruvate), pre-warmed to 37°C
- Sterile, 10 cm tissue culture plates (one each for the lentiviral construct, positive control, and negative control)
- Sterile, tissue culture supplies
- 15 mL sterile, capped, conical tubes
- Cryovials
- CO_2 humidified incubator set at 37°C
- Centrifuge capable of 2,000 × g
- *Optional:* Millex-HV 0.45 µm PVDF filters (Millipore, cat. no. SLHVR25LS) or equivalent, to filter viral supernatants
- *Optional:* pLenti control vector containing EmGFP (sold separately; see page 60)

Materials supplied with the kit:

- ViraPower™ Packaging Mix
- pLenti3.3/TR repressor plasmid
- pLenti6.3/TO/V5-GW/*lac*Z control vector (at 0.5 µg/µL in TE, pH 8.0)
- Lipofectamine™ 2000 transfection reagent (mix gently before use)

Continued on next page

Forward Transfection Procedure

If you are a **first time user**, follow the procedure below to co-transfect 293FT cells. For information on positive controls, see page 7. We recommend including a negative control (no DNA, no Lipofectamine™ 2000) in your experiment to help you evaluate your results.

Day 1:

1. The day before transfection, plate 293FT cells in a 10 cm tissue culture plate so that they are 90–95% confluent on the day of transfection (i.e., 5×10^6 cells in 10 mL of growth medium containing serum, see previous page). **Do not include antibiotics in the medium.** Incubate cells overnight at 37°C in a humidified 5% CO_2 incubator.

Day 2:

2. On the day of transfection, remove and discard the culture medium from the 293FT cells and replace with 5 mL of growth medium containing serum (*i.e.*, D-MEM containing 10% FBS, 4 mM L-Glutamine, 0.1 mM MEM Non-Essential Amino Acids, and 1 mM MEM sodium pyruvate). **Do not use antibiotics in the medium.**

   Note: You may also use 5 mL of Opti-MEM® I medium supplemented with 2–5% FBS.

3. **For each transfection sample**, prepare DNA-Lipofectamine™ 2000 complexes as follows:

   a. In a sterile 5 mL tube, dilute 9 µg of the ViraPower™ Packaging Mix and 3 µg of your pLenti plasmid DNA (12 µg total) in 1.5 mL of Opti-MEM® I medium without serum. Mix gently.

   b. In a separate, sterile 5 mL tube, dilute 36 µL Lipofectamine™ 2000 (mix gently before use) in 1.5 mL of Opti-MEM® I medium without serum. Mix gently and incubate for 5 minutes at room temperature.

      Note: Proceed to Step c within 25 minutes.

   c. After incubation, combine the diluted DNA (Step a) with the diluted Lipofectamine™ 2000 (Step b). Mix gently.

   d. Incubate for 20 minutes at room temperature to allow the DNA-Lipofectamine™ 2000 complexes to form. The solution may appear cloudy, but this will not impede the transfection.

      Note: The complexes are stable for 6 hours at room temperature.

4. Add all the DNA-Lipofectamine™ 2000 complexes dropwise to the culture plates containing 293FT cells (Step 2). Mix gently by rocking the plate back and forth. Incubate the cells overnight at 37°C in a humidified 5% CO_2 incubator.

Procedure continued on next page

Continued on next page

Forward Transfection Procedure, continued

Procedure continued from previous page

Day 3:

5. Remove the cell culture plate containing the 293FT cells with DNA-Lipofectamine™ complexes from the incubator. Remove and discard the medium containing the DNA-Lipofectamine™ 2000 complexes and replace with 10 mL complete culture medium **without antibiotics**.
6. Incubate cells for 24–48 hours at 37°C in a humidified 5% CO_2 incubator. (Minimal differences in viral yield are observed whether supernatants are collected at either 48 or 72 hours post-transfection).

   Note: Expression of the VSV G glycoprotein causes 293FT cells to fuse, resulting in the appearance of large, multinucleated cells known as syncytia. This morphological change is normal and does not affect production of the lentivirus.

Day 5 or 6:

7. Post-transfection (Day 5 or 6), harvest virus-containing supernatants by removing and transferring the medium into a 15 mL sterile, capped, conical tube.

   Caution: You are working with infectious virus at this stage. Follow recommended guidelines for working with BL-2 organisms (refer to page 4).
8. Centrifuge supernatants at 2,000 × g for 15 minutes at 4°C to pellet debris.
9. *Optional:* Filter the viral supernatants through a Millex-HV 0.45 µm or equivalent PVDF filter (see **Note**, page 16).
10. Pipet viral supernatants into cryovials in 1 mL aliquots.
11. Store viral stocks at –80°C. Proceed to **Titering Your Lentiviral Stock**, page 18.

Signs of Lentivirus Production in 293FT Cells

During lentivirus production, transfected 293FT cells go through the following morphological changes:

- Expression of the VSV G glycoprotein causes 293FT cells to fuse, resulting in the appearance of large, **multinucleated** cells known as syncytia. Appearance of syncytia is a good sign of virus production.
- The cells start to look like balloons.
- They often, but not always, lift off from the surface of the culture dish.
- Untransfected 293FT cells leave empty spaces on the surface of the culture dish and pile up at other spots of the dish.

For time-course images of 293FT cells transfected with the Vivid Colors™ pLenti6.3/V5-GW/EmGFP expression control vector, see **Change in 293FT Morphology** in the **Appendix**, pages 43–46.

Continued on next page

Reverse Transfection Procedure

If you are an **experienced user**, you may use the rapid, reverse transfection procedure to co-transfect 293FT cells. For information on positive controls, see page 7. We recommend including a negative control (no DNA, no Lipofectamine™ 2000) in your experiment to help you evaluate your results. You need 6×10^6 293FT cells for each sample.

Day 1:

1. Prepare DNA-Lipofectamine™ 2000 complexes **for each transfection sample** as follows:
   a. In a sterile 5 mL tube, dilute 9 µg of the ViraPower™ Packaging Mix and 3 µg of pLenti plasmid DNA (12 µg total) in 1.5 mL of Opti-MEM® I medium without serum. Mix gently.
   b. In a separate sterile 5 mL tube, dilute 36 µL Lipofectamine™ 2000 (mix gently before use) in 1.5 mL of Opti-MEM® I medium without serum. Mix gently and incubate for 5 minutes at room temperature.
   c. After incubation, combine the diluted DNA (Step a) with the diluted Lipofectamine™ 2000 (Step b). Mix gently.
      Note: Proceed to Step c within 25 minutes.
   d. Incubate for 20 minutes at room temperature to allow the DNA-Lipofectamine™ 2000 complexes to form. The solution may appear cloudy, but this will not impede the transfection.
      Note: The complexes are stable for 6 hours at room temperature.
2. While DNA-lipid complexes are forming, trypsinize and count the 293FT cells. Resuspend the cells at a density of 1.2×10^6 cells/mL in growth medium containing serum (i.e., D-MEM containing 10% FBS, 4 mM L-Glutamine, 0.1 mM MEM Non-Essential Amino Acids, and 1 mM MEM sodium pyruvate). **Do not include antibiotics in the medium.**
   Note: You may also use 5 mL of Opti-MEM® I medium supplemented with 2–5% FBS.
3. Add the DNA-Lipofectamine™ 2000 complexes (Step 1d) to a 10 cm tissue culture plate containing 5 mL of growth medium (or Opti-MEM® I medium) containing serum. **Do not include antibiotics in the medium.**
4. Add 5 mL of the 293FT cell suspension from Step 2 (6×10^6 total cells) to the plate containing media and DNA-Lipofectamine™ 2000 complexes (Step 3). Mix gently by rocking the plate back and forth. Incubate cells overnight at 37°C in a humidified 5% CO_2 incubator.

Day 2:

5. The next day (Day 2), remove and discard the medium containing the DNA-Lipofectamine™ 2000 complexes and replace with 10 mL complete culture medium **without antibiotics**.
6. Incubate cells for 24–48 hours at 37°C in a humidified 5% CO_2 incubator. (Minimal differences in viral yield are observed whether supernatants are collected at either 48 or 72 hours posttransfection).
   Note: Expression of the VSV G glycoprotein causes 293FT cells to fuse, resulting in the appearance of large, multinucleated cells known as syncytia. This morphological change is normal and does not affect production of the lentivirus.

Procedure continued on next page

Continued on next page

Reverse Transfection Procedure, continued

Procedure continued from previous page

Day 4 or 5:

7. Posttransfection (Day 4 or 5), harvest virus-containing supernatants by removing and placing the medium into a 15 mL sterile, capped, conical tube.
   Caution: You are working with infectious virus at this stage. Follow recommended guidelines for working with BL-2 organisms (refer to page 4).
8. Centrifuge supernatants at 2,000 × g for 15 minutes at 4°C to pellet debris.
9. *Optional:* Filter the viral supernatants through a Millex-HV 0.45 µm or equivalent PVDF filter (see **Note** below).
10. Pipet viral supernatants into cryovials in 1 mL aliquots.
11. Store viral stocks at –80°C. Proceed to **Titering Your Lentiviral Stock**, page 18.

Signs of Lentivirus Production in 293FT Cells

During lentivirus production, transfected 293FT cells go through the following morphological changes:

- Expression of the VSV G glycoprotein causes 293FT cells to fuse, resulting in the appearance of large, **multinucleated** cells known as syncytia. Appearance of syncytia is a good sign of virus production.
- The cells start to look like balloons.
- They often, but not always, lift off from the surface of the culture dish.
- Untransfected 293FT cells leave empty spaces on the surface of the culture dish and pile up at other spots of the dish.

For time-course images of 293FT cells transfected with the Vivid Colors™ pLenti6.3/V5-GW/EmGFP expression control vector, see **Change in 293FT Morphology** in the **Appendix**, pages 43–46.

Note

It should be possible to use the new ViraPower™ HiPerform™ T-REx™ lentiviral vector constructs for *in vivo* applications, however, we have not yet tested the new constructs *in vivo*.

If you plan to use your lentiviral construct for *in vivo* applications, we recommend filtering your viral supernatant through a sterile, 0.45 µm low protein binding filter after the low-speed centrifugation step (Step 8, page 14 and Step 8, above) to remove any remaining cellular debris. We recommend using Millex-HV 0.45 µm PVDF filters (Millipore, Catalog no. SLHVR25LS) for filtration.

If you wish to concentrate your viral stock to obtain a higher titer, perform the filtration step first before concentrating your viral stock.

Continued on next page

Concentrating Virus

It is possible to concentrate VSV-G pseudotyped lentiviruses using a variety of methods without significantly affecting their ability to transduce cells. If your cell transduction experiment requires that you use a relatively high Multiplicity of Infection (MOI), you may wish to concentrate your virus before titering and proceeding to transduction. For details and guidelines to concentrate your virus supernatant by ultracentrifugation, refer to published reference sources (Yee, 1999).

Long-Term Storage

Store viral stocks at –80°C in cryovials for long-term storage. We do **not** recommend repeated freezing and thawing as it may result in loss of viral titer. When stored properly, viral stocks of an appropriate titer are suitable for use for up to one year. After long-term storage, we recommend retitering your viral stocks before transducing your mammalian cell line of interest.

Scaling Up Virus Production

It is possible to scale up the co-transfection experiment to produce a larger volume of lentivirus, if desired. For example, we have scaled up the co-transfection experiment from a 10 cm plate to a T-175 flask, and harvested up to 30 mL of viral supernatant. If you wish to scale up your co-transfection, increase the number of cells plated and the amounts of DNA, Lipofectamine™ 2000, and medium used in proportion to the difference in surface area of the culture vessel.

Titering Your Lentiviral Stock

Introduction

Before proceeding to transduction and expression experiments, we highly recommend determining the titer of your lentiviral stock. While this procedure is not required for some applications, it is necessary for:

- Controlling the number of integrated copies of the lentivirus

  and

- generating reproducible expression results.

Factors Affecting Viral Titer

- **The size of your gene of interest:** Viral titer decreases as the size of the insert increases. We have determined that virus titer drops approximately 2-fold for each kb over 4 kb of insert size. To produce lentivirus with an insert of > 4 kb, you need to concentrate the virus to obtain a suitable titer (see page 17). The size of the wild-type HIV genome is approximately 10 kb. Because the size of the elements required for expression from pLenti vectors total approximately 4–4.4 kb, the size of your insert should not exceed 5.6 kb.
- **The characteristics of the cell line used for tittering**: We recommend using the human fibrosarcoma line HT1080 (see **Selecting a Cell Line for Titering**, below). However, other cell lines may be used. In general, cells used for titering lentivirus should be an adherent, non-migratory cell line, and exhibit a doubling time in the range of 18–25 hours.
- **The age of your lentiviral stock:** Viral titers may decrease with long-term (>1 year) storage at –80°C. If your lentiviral stock has been stored for longer than 6 months, we recommend titering your lentiviral stock prior to use.
- **The number of freeze/thaw cycles:** Viral titers can decrease as much as 10% with each freeze/thaw cycle.
- **Improper storage of your lentiviral stock:** Store lentiviral stocks in cryovials at –80°C.

Selecting a Cell Line for Titering

We strongly recommend the human fibrosarcoma line HT1080 (ATCC, Cat no. CCL-121) as the "gold standard" for reproducibly titering lentivirus. However, you may wish to use the same mammalian cell line to titer your lentiviral stocks as you will use to perform your expression studies (e.g., if you are performing expression studies in a dividing cell line or a non-primary cell line). If you have more than one lentiviral construct, we recommend that you titer all of the lentiviral constructs using the same mammalian cell line.

Note

The titer of a lentiviral construct may vary depending on the chosen cell line. When titering more than one lentiviral construct, we recommend using the same mammalian cell line to titer all of the lentiviral constructs.

Continued on next page

Titering Your Lentiviral Stock, Continued

Antibiotic Selection

The pLenti6.3/TO/V5-DEST and pLenti6.3/TO/V5-GW/*lac*Z expression constructs contain the Blasticidin resistance gene (bsd) (Kimura et al., 1994) and the pLenti3.3/TR repressor plasmid contains the neomycin resistance gene to allow for Blasticidin (Takeuchi et al., 1958; Yamaguchi et al., 1965) or Geneticin® (Southern & Berg, 1982) selection, respectively, of mammalian cells that have stably transduced the lentiviral construct.

For more information on preparing and handling Blasticidin and Geneticin®, and on determining the sensitivity of your cell line to these antibiotics, refer to the **Appendix**, pages 41 and 42, respectively.

Note: Blasticidin and Geneticin® are supplied with the kit and are also available separately from Invitrogen (see page 60).

Using Polybrene® During Transduction

Lentivirus transduction may be enhanced if cells are transduced in the presence of hexadimethrine bromide (Polybrene®, Sigma Cat. no. H9268). For best results, we recommend performing transduction in the presence of Polybrene®. Note, however, that some cells are sensitive to Polybrene® (e.g., primary neurons).

Before performing any transduction experiments, test your cell line for sensitivity to Polybrene® at a range of 0–10 µg/mL. If your cells are sensitive to Polybrene® (e.g., exhibit toxicity or phenotypic changes), do not add Polybrene® during transduction. In this case, cells should still be successfully transduced with your lentivirus.

Polybrene® is a registered trademark of Abbott Laboratories.

Preparing and Storing Polybrene®

Follow the instructions below to prepare Polybrene®:

1. Prepare a 6 mg/mL stock solution in deionized, sterile water.
2. Filter-sterilize and dispense 1 mL aliquots into sterile microcentrifuge tubes.
3. You may store the working stock at 4°C for up to 2 weeks. Store at –20°C for long-term storage (up to 1 year). Do not freeze/thaw the stock solution more than 3 times as this may result in loss of activity.

Experimental Outline

To determine the titer of a lentiviral stock:

1. Prepare 10-fold serial dilutions of your lentiviral stock.
2. Transduce the different dilutions of lentivirus into the mammalian cell line of choice in the presence of Polybrene®.
3. Select for stably transduced cells using the appropriate selection agent.
4. Stain and count the number of antibiotic-resistant colonies in each dilution.

See next page for a detailed protocol for titering your lentiviral stock.

Continued on next page

Titering Your Lentiviral Stock, Continued

Remember that you are working with media containing infectious virus. Follow the recommended Federal and institutional guidelines for working with BL-2 organisms.

- Perform all manipulations within a certified biosafety cabinet.
- Treat media containing virus with bleach.
- Treat used pipettes, pipette tips, and other tissue culture supplies with bleach and dispose of as biohazardous waste.
- Wear gloves, a laboratory coat, and safety glasses or goggles when handling viral stocks and media containing virus.

Materials Needed

- Your Lenti6.3/TO/V5-DEST lentiviral stock (store at –80°C until use)
- Your Lenti3.3/TR lentiviral stock (store at –80°C until use)
- Your Lenti6.3/TO/V5-GW/*lacZ* lentiviral stock (if produced; store at –80°C until use)
- Adherent mammalian cell line of choice
- Complete culture medium for your cell line
- 6 mg/mL Polybrene®, if desired
- 6-well tissue culture plates (for every lentiviral stock you are titering, **at least** one 6-well plate for one mock well plus five dilutions)
- Blasticidin or Geneticin®, as appropriate for selection
- Crystal violet (Sigma, Cat. no. C3886; prepare a 1% crystal violet solution in 10% ethanol)
- Phosphate-Buffered Saline (PBS) (see page 60)

Continued on next page

Titering Your Lentiviral Stock Using Blasticidin, Continued

Transduction and Titering Procedure

You need **at least** one 6-well plate for every lentiviral stock you are titering (one mock well plus five dilutions).

Day 1:

1. The day before transduction, trypsinize and count the cells, plating them in a 6-well plate such that they will be 30–50% confluent at the time of transduction. Incubate cells at 37°C overnight.

   Note: When using HT1080 cells, we usually plate 2×10^5 cells per well in a 6-well plate.

Day 2:

2. On the day of transduction, thaw your lentiviral stock and prepare 10-fold serial dilutions ranging from 10^{-2} to 10^{-6}. For each dilution, dilute the lentiviral construct into complete culture medium to a final volume of 1 mL. **Do not vortex.**

   Note: You may prepare a wider range of serial dilutions (10^{-2} to 10^{-8}), if desired.

3. Remove the culture medium from the cells. Mix each dilution gently by inversion and add to one well of cells (total volume = 1 mL).
4. Add Polybrene® (if desired) to each well to a final concentration of 6 µg/mL. Swirl the plate gently to mix. Incubate at 37°C overnight.

Day 3:

5. The following day, remove the media containing virus and replace with 2 mL of complete culture medium.

Day 4:

6. The following day, remove the medium and replace with complete culture medium containing the appropriate amount of Blasticidin (for Lenti6.3/TO/V5-DEST and Lenti6.3/TO/V5-GW/*lacZ* lentiviral stock) or Geneticin® (for Lenti3.3/TR lentiviral stock) to select for stably transduced cells.

   Note: Because Geneticin® is not very effective at high cell densities, it might be necessary to dilute your cells to select for stable transductants.

7. Replace medium with fresh medium containing antibiotic every 2–3 days.

Day 14–16:

8. After 10–12 days of selection (day 14–16), you should see no live cells in the mock well and discrete antibiotic-resistant colonies in one or more of the dilution wells. Remove the medium and wash the cells twice with PBS.
9. Add crystal violet solution (1 mL for 6-well dish; 5 mL for a 10 cm plate) and incubate for 10 minutes at room temperature.
10. Remove the crystal violet stain and wash the cells twice with PBS.
11. Count the blue-stained colonies and determine the titer of your lentiviral stock.

Expected Titer

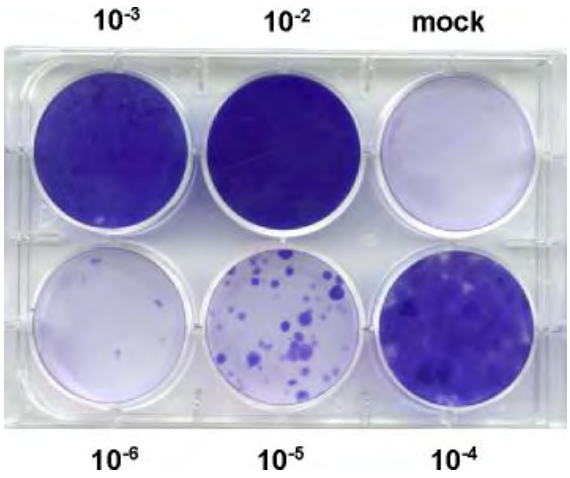
When titering pLenti lentiviral stocks using HT1080 cells, we generally obtain titers ranging from 1×10^5–5×10^5 transducing units (TU)/mL (for unconcentrated virus) up to 2×10^7 TU/mL (for concentrated virus).

Continued on next page

Titering Your Lentiviral Stock Using Blasticidin, Continued

Example of Expected Results

In this experiment, a Lenti6.3/V5-GW/*lacZ* lentiviral stock was generated using the protocol on pages 13–14 and was concentrated by ultracentrifugation. HT1080 cells were transduced with 10-fold serial dilutions of the lentiviral supernatant (10^{-2} to 10^{-6} dilutions) or untransduced (mock) following the protocol on page 21. At 48 hours post-transduction, the cells were placed under Blasticidin selection (10 µg/mL). After 10 days of selection, the cells were stained with crystal violet (see plate below), and colonies were counted.



In the plate above, the colony counts were:

- Mock: no colonies
- 10^{-2} dilution: confluent; undeterminable
- 10^{-3} dilution: confluent; undeterminable
- 10^{-4} dilution: confluent; undeterminable
- 10^{-5} dilution: 46
- 10^{-6} dilution: 5

Thus, the titer of this concentrated lentiviral stock is 4.8×10^6 TU/mL (i.e., average of 46×10^5 and 5×10^6).

Next Steps

It is important to note that user experience, the nature of the gene, and vector backbone may affect virus titer. If the titer of your unconcentrated virus is suitable (i.e., 1×10^5 TU/mL or higher), proceed to transducing your cells with your lentivirus stocks. If the titer of your concentrated lentiviral stock is less than 1×10^5 TU/mL, we recommend producing a new lentiviral stock. See **Troubleshooting** (page 36) for more tips and guidelines to optimize your viral yield.

General Considerations for Transduction and Expression

Introduction

After you have generated lentiviral stocks with suitable titers, you are ready to transduce the lentiviral constructs into the mammalian cell line of choice and assay for expression of your recombinant protein. This section provides general guidelines to help you design your transduction and expression experiment. We recommend that you read through this section before beginning.

Important

Each lentiviral construct contains a deletion in the 3′ LTR that leads to self-inactivation of the lentivirus after transduction into mammalian cells. After it is integrated into the genome, the lentivirus can no longer produce packageable virus.

Factors to Consider When Designing Your Expression Experiment

When designing your expression experiment, consider the factors below:

- Options available to express your recombinant protein
- Whether to express the recombinant protein transiently or stably
- How much Tet repressor to express in your mammalian cell line
- How much virus to use for transduction (i.e., MOI)
- How much tetracycline to use for induction

Each of these factors is discussed further in this section.

Expression Options

A number of options exist to express your gene of interest in the mammalian cell line of choice. Choose the option that best fits your needs.

Option	Procedure	Benefit
1	"Co-transduce" the Lenti3.3/TR and Lenti6.3/TO/V5-DEST lentiviral constructs into mammalian cells (see pages 27–30)	Perform regulated expression experiments with a single transduction
2	Transduce your mammalian cell line with the Lenti3.3/TR lentiviral construct and generate a stable cell line. Use this ViraPower™ T-REx™ cell line as the host for the Lenti6.3/TO/V5-DEST lentiviral construct (see pages 32–35)	Perform regulated expression experiments with multiple expression constructs using a cell line that consistently expresses the same amount of Tet repressor
3	Transduce your mammalian cell line with the Lenti6.3/TO/V5-DEST lentivirus only	Constitutively express the gene of interest

For optimal results, we recommend generating a stable ViraPower™ T-REx™ cell line, then using this cell line as the host for your Lenti6.3/TO/V5-DEST expression construct (i.e., Option 2, above). We particularly recommend this option if you want to perform regulated expression experiments with several expression constructs in the same mammalian cell line. For guidelines and instructions to generate a ViraPower™ T-REx™ cell line, see **Generating a ViraPower™ T-REx™ Host Cell Line**, page 32.

Continued on next page

General Considerations for Transduction and Expression, Continued

Transient vs. Stable Expression

When designing your expression experiment, consider how to assay for expression of your gene of interest. After you have transduced your Lenti6.3/TO/V5-DEST lentiviral construct into mammalian cells, you may:

- Pool a heterogeneous population of cells and test for expression of your recombinant protein directly after transduction (i.e., "transient" expression). Note that you must wait for a minimum of 48–72 hours after transduction and induction (for expression Options 1 and 2, previous page) before harvesting your cells to allow expressed protein to accumulate in transduced cells.
- Select for stably transduced cells using Blasticidin. This requires a minimum of 10–12 days after transduction, but allows generation of clonal cell lines that stably express the gene of interest. Recombinant protein expression are tetracycline-regulated (for expression Options 1 and 2, previous page) or constitutive (for expression Option 3, previous page).

Determining Antibiotic Sensitivity for Your Cell Line

To select for stably transduced cells expressing Lenti3.3/TR or the Lenti6.3/TO/V5-DEST lentiviral construct, first determine the minimum concentration of the appropriate antibiotic that is required to kill your untransduced mammalian cell line (i.e., perform a kill curve experiment). For guidelines to perform a kill curve experiment, see pages 41–42.

If you have titered your Lenti3.3/TR and/or Lenti6.3/TO/V5-DEST construct in the same mammalian cell line that you are using to generate a stable cell line, use for selection the same concentration of the antibiotic that you used for titering.

Expression of Tet Repressor (TetR)

Because tetracycline-regulated expression in the ViraPower™ HiPerform™ T-REx™ Gateway® Expression System is based on a repression/derepression mechanism, the amount of Tet repressor that is expressed in the host cell line from the Lenti3.3/TR lentiviral construct determines the level of transcriptional repression of the Tet operator sequences in the Lenti6.3/TO/V5-DEST lentiviral construct. **Tet repressor levels need to be sufficiently high to suitably repress basal level transcription.** When performing co-transduction experiments, we generally do the following to maximize Tet repressor expression levels:

- Transduce the Lenti3.3/TR construct into mammalian cells and wait for 24 hours before transducing the Lenti6.3/TO/V5-DEST construct to allow time for the Tet repressor protein to be expressed
- Transduce the Lenti3.3/TR construct into mammalian at a higher MOI (see next page) than the Lenti6.3/TO/V5-DEST construct

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General Considerations for Transduction and Expression, Continued

Multiplicity of Infection (MOI)

To obtain optimal expression of Tet repressor or your gene of interest, you need to transduce the lentiviral construct into your mammalian cell line of choice using a suitable MOI. MOI is defined as the number of virus particles per cell and generally correlates with the number of integration events, and as a result, expression. Typically, expression levels increase as the MOI increases.

Determining the Optimal MOI

A number of factors can influence determination of an optimal MOI including:

- The nature of your mammalian cell line (e.g., non-dividing vs. dividing cell type; see **Note** below)
- The transduction efficiency of your mammalian cell line
- The nature of your gene of interest
- The procedure you are using to express your gene of interest (i.e., see **Expression Options**, page 23).

If you are transducing the Lenti3.3/TR and/or your Lenti6.3/TO/V5-DEST lentiviral construct into the mammalian cell line of choice for the first time, we recommend using a range of MOIs (e.g., 0, 1, 5, 10, 50) to determine the MOI required to obtain the optimal expression of the gene of interest.

Note: If you are using Expression Options 1 or 2, recommended MOIs to use for transduction are provided with each procedure. Use the recommended MOIs as a starting point for your experiments, and optimize as desired.

Note

In general, non-dividing cell types transduce lentiviral constructs less efficiently than actively dividing cell lines. If you are transducing your lentiviral construct into a non-dividing cell type, you may need to increase the MOI to achieve optimal gene expression levels.

Positive Control

If you have packaged the control Lenti6.3/TO/V5-GW/*lacZ* lentiviral construct, we recommend using the lentiviral stock to help you determine the optimal MOI for your particular cell line. After transducing the control lentivirus into your mammalian cell line of choice (i.e., native host or ViraPower™ T-REx™ host cell line), you may easily assay for constitutive or induced β-galactosidase expression, as appropriate (see page 31 for more information).

Tetracycline

Tetracycline (MW = 444.4) is commonly used as a broad spectrum antibiotic and acts to inhibit translation by blocking polypeptide chain elongation in bacteria. In the ViraPower™ HiPerform™ T-REx™ Gateway® Expression System, tetracycline functions as an inducing agent to regulate transcription of the gene of interest from the Lenti6.3/TO/V5-DEST lentiviral construct.

Tetracycline induces transcription by binding to the Tet repressor homodimer, causing the repressor to undergo a conformational change that renders it unable to bind to the Tet operator in the CMV/TO promoter. The association constant of tetracycline to the Tet repressor is 3×10^9 M^{-1} (Takahashi *et al.*, 1991).

Continued on next page

General Considerations for Transduction and Expression, Continued

Using Tetracycline

To induce transcription of the gene of interest in mammalian cells, we generally add tetracycline to a final concentration of 1 µg/mL in complete growth medium. If desired, you may vary the concentration of tetracycline used for induction from 0.001 µg/mL to 1 µg/mL to modulate expression of the gene of interest.

Note: The concentrations of tetracycline used for induction in the ViraPower™ HiPerform™ T-REx™ Gateway® Expression System are generally not high enough to be toxic to mammalian cells.

Follow the guidelines below when handling tetracycline.

- Tetracycline is light sensitive. Store the stock solution at –20°C, protected from light. Prepare medium containing tetracycline immediately before use.
- Tetracycline is toxic. Do not ingest solutions containing the drug. If handling the powdered form, do not inhale.
- Wear gloves, a laboratory coat, and safety glasses or goggles when handling tetracycline and tetracycline-containing solutions.

Tetracycline in Fetal Bovine Serum

When culturing cells in medium containing fetal bovine serum (FBS), note that many lots of FBS contain tetracycline as FBS is generally isolated from cows that have been fed a diet containing tetracycline. If you culture your mammalian cells in medium containing FBS that is not reduced in tetracycline, you may observe some basal expression of your gene of interest in the absence of tetracycline.

We generally culture our mammalian cells in medium containing FBS that may not be reduced in tetracycline, and have observed low basal expression of target genes in the absence of tetracycline. Depending on your application (e.g., if expressing a toxic protein), you may wish to culture your cells in tetracycline-tested FBS. For more information, consult the supplier of your FBS.

Important

Viral supernatants are generated by harvesting spent media containing virus from the 293FT producer cells. Spent media lacks nutrients and may contain some toxic waste products. If you are using a large volume of viral supernatant to transduce your mammalian cell line (e.g., 1 mL of viral supernatant per well in a 6-well plate), the growth characteristics or morphology of the cells may be affected during transduction. These effects are generally alleviated after transduction when the media is replaced with fresh, complete media.

Concentrating Virus

It is possible to concentrate VSV-G pseudotyped lentiviruses using a variety of methods without significantly affecting their transducibility. If the titer of your lentiviral stock is relatively low (less than 1×10^5 TU/mL) and your experiment requires that you use a large volume of viral supernatant (e.g., a relatively high MOI), concentrate your virus before proceeding to transduction. For details and guidelines for concentrating your virus, refer to published reference sources (Yee, 1999).

Co-Transduction and Tetracycline-Regulated Expression

Introduction

We recommend using the co-transfection procedure if you have a single Lenti6.3/TO/V5-DEST lentiviral construct and you wish to verify that your gene of interest can be inducibly expressed in the mammalian cell line of interest.

If you have multiple Lenti6.3/TO/V5-DEST lentiviral constructs, we recommend first generating a ViraPower™ T-REx™ cell line expressing the Tet repressor, and using this cell line as the host for your lentiviral constructs (see **Generating a ViraPower™ T-REx™ Host Cell Line**, pages 32–35 for details).

Note: If you wish to constitutively express your gene of interest, simply transduce the Lenti6.3/TO/V5-DEST construct alone into cells at a suitable MOI.

When performing the co-transduction procedure, use Lenti3.3/TR and Lenti6.3/TO/V5-DEST lentiviral stocks of known titer. Optimal expression results are generally obtained (i.e., low basal and high inducible expression levels) when the Lenti3.3/TR construct is transduced into mammalian cells at a higher MOI than the Lenti6.3/TO/V5-DEST construct (see **MOI to Use for Transduction**, next page).

Depending on the cell line used and the nature of your gene of interest, vary the ratio of Lenti3.3/TR lentivirus:Lenti6.3/TO/V5-DEST lentivirus transduced into host cells to optimize basal and induced recombinant protein expression levels. Optimization is best accomplished when the titer of each lentiviral stock is known.

Experimental Outline

To express the gene of interest using the co-transduction procedure:

1. Transduce the Lenti3.3/TR lentiviral construct into mammalian cells at a suitable MOI (e.g., MOI = 10).
2. Incubate cells for 24 hours, then transduce the Lenti3.3/TR-containing cells with the Lenti6.3/TO/V5-DEST lentiviral construct at a slightly lower MOI (e.g., MOI = 1–5).
3. Incubate the cells for 24 hours, and remove the medium-containing virus.
4. Incubate the cells for 24 hours, and add tetracycline to induce expression of the gene of interest. Alternatively, select for stably transduced cells using Blasticidin and Geneticin®. After you generate stable cell lines, add tetracycline to induce expression of the gene of interest.

Important

When performing the co-transduction procedure, you **must** transduce the Lenti3.3/TR lentiviral construct into mammalian cells **before** transducing the Lenti6.3/TO/V5-DEST expression construct to enable tetracycline-regulated expression of the gene of interest.

We generally wait at least 24 hours after transducing the Lenti3.3/TR construct before transducing the Lenti6.3/TO/V5-DEST construct to allow time for the Tet repressor to be expressed.

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Co-Transduction and Tetracycline-Regulated Expression, Continued

MOI to Use for Transduction

Transduce the Lenti3.3/TR and Lenti6.3/TO/V5-DEST lentiviral constructs into your mammalian cell line at any suitable MOI (see **Determining the Optimal MOI**, page 25). To sufficiently repress basal transcription of the gene of interest and still obtain maximal levels of tetracycline-induced expression, we recommend transducing the Lenti3.3/TR construct into cells at a higher MOI than the Lenti6.3/TO/V5-DEST construct.

As a starting point, we recommend transducing the Lenti3.3/TR construct into cells at an MOI of 10, and transducing the Lenti6.3/TO/V5-DEST construct into cells at an MOI of 1 to 5.

You may optimize basal and tetracycline-induced expression levels by varying the MOI of the Lenti3.3/TR and/or Lenti6.3/TO/V5-DEST lentiviruses.

Materials Needed

- Titered Lenti3.3/TR lentiviral stock (store at –80°C until use)
- Titered Lenti6.3/TO/V5-DEST lentiviral stock (store at –80°C until use)
- Mammalian cell line of choice
- Complete culture medium for your cell line
- 6 mg/mL Polybrene®, if desired
- Appropriately sized tissue culture plates for your application
- 10 mg/mL tetracycline (supplied with the kit; store **protected from light**)
- 10 mg/mL Blasticidin stock solution (if selecting for stably transduced Lenti6.3/TO/V5-DEST cells)
- Geneticin® stock solution (if selecting for stably transduced Lenti3.3/TR cells)

Continued on next page

Co-Transduction and Tetracycline-Regulated Expression,
Continued

Co-Transduction Procedure

Follow the procedure below for co-transducing your cells with Lenti3.3/TR and Lenti6.3/TO/V5-DEST lentiviral constructs to assay for tetracycline-regulated expression of your gene of interest. We recommend including a negative control (mock transduction) to help you evaluate your results. If you are selecting for stable cell lines, include two negative control samples, one for Blasticidin selection and the other for Geneticin® selection.

1. Plate cells in complete growth media as appropriate for your application.

Day 1:

2. On the day of transduction (Day 1), thaw the Lenti3.3/TR lentiviral stock and dilute (if necessary) the appropriate amount of virus (at a suitable MOI; recommended MOI = 10) into fresh complete culture medium. Keep the total volume of medium containing virus as low as possible to maximize transduction efficiency. **Do not vortex.**
3. Remove the culture medium from the cells. Mix the medium containing virus gently by pipetting and add to the cells.
4. Add Polybrene® (if desired) to a final concentration of 6 μg/mL. Swirl the plate gently to mix. Incubate at 37°C overnight.

Day 2:

5. Twenty-four hours following transduction of Lenti3.3/TR virus (Day 2), thaw the Lenti6.3/TO/V5-DEST lentiviral stock and dilute (if necessary) the appropriate amount of virus (at a suitable MOI; recommended MOI = 1 to 5) into fresh complete medium. Keep the total volume of medium containing virus as low as possible to maximize transduction efficiency. **Do not vortex.**
6. Remove the culture medium containing Lenti3.3/TR virus from the cells. Mix the medium containing Lenti6.3/TO/V5-DEST virus gently by pipetting and add to the Lenti3.3/TR virus-containing cells.
7. Add Polybrene® (if desired) to a final concentration of 6 μg/mL. Swirl the plate gently to mix. Incubate at 37°C overnight.

Procedure continued on next page

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Co-Transduction and Tetracycline-Regulated Expression, Continued

Co-Transduction Procedure, continued

Procedure continued from previous page

Day 3:

8. Twenty-four hours following transduction of Lenti6.3/TO/V5-DEST virus (Day 3), perform one of the following:

- **Transient expression experiments:** Remove the medium containing virus and replace with fresh, complete medium containing 1 µg/mL tetracycline. Incubate the cells at 37°C for 24–48 hours before assaying for expression of your recombinant protein. To assay the cells at a later time, continue to culture the cells, or replate them into larger-sized tissue culture formats in medium containing tetracycline.
- **Stable cell lines:** Remove the medium and replace with fresh, complete medium containing the appropriate amount of Blasticidin. Incubate the cells at 37°C for 24 hours, then trypsinize and replate them into a larger-sized tissue culture format in fresh, complete medium containing Blasticidin and Geneticin®. Proceed to Step 9, below.

  Example: If transducing cells in a 6-well format, trypsinize and replate cells into a 10 cm tissue culture plate before performing Blasticidin and Geneticin® selection.

For stable cell lines only

9. Replace medium with fresh medium containing Blasticidin and Geneticin® every 2–3 days until you can identify Blasticidin- and Geneticin®-resistant colonies (generally 10–14 days after selection).

   Note: Transducing cells with Lenti3.3/TR and Lenti6.3/TO/V5-DEST lentivirus at a high MOI results in most of the cells being Blasticidin- and Geneticin®-resistant. In this case, you may not be able to see distinct Blasticidin- and Geneticin®-resistant colonies when performing stable selection. You may also not see many non-transduced cells (i.e., dead cells).

10. Pick at least 10 Blasticidin- and Geneticin®-resistant colonies (see **Note** below) and expand each clone. Alternatively, you may pool the heterogeneous population of Blasticidin- and Geneticin®-resistant cells.
11. Induce expression of the gene of interest by adding tetracycline to a final concentration of 1 µg/mL. Wait for the appropriate length of time (e.g., 24–48 hours) before assaying for your recombinant protein.

Note

Integration of the lentivirus into the genome is random. Depending upon the influence of the surrounding genomic sequences at the integration site, you may see varying levels of gene expression from different Blasticidin- and Geneticin®-resistant clones. For further studies, we recommend testing at least 10 Blasticidin- and Geneticin®-resistant clones, and selecting the clone that provides the lowest level of basal expression and the highest level of induced gene expression.

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Detecting Recombinant Protein

To detect expression of your recombinant fusion protein, you may perform:

- Western blot analysis using the Anti-V5, Anti-V5-HRP, or Anti-V5-AP antibodies available from Invitrogen or an antibody to your protein
- Immunofluorescence using the Anti-V5-FITC antibody available from Invitrogen
- Functional analysis

For more information about the Anti-V5 antibodies, refer to www.invitrogen.com or contact Technical Support (see page 62). See page 61 for ordering information.

Assaying for β-galactosidase

If you use the Lenti6.3/TO/V5-GW/*lacZ* positive control lentiviral construct in a co-transduction experiment with Lenti3.3/TR, you may assay for β-galactosidase expression by Western blot analysis or activity assay using cell-free lysates (Miller, 1972). Invitrogen offers the β-Gal Assay Kit (see page 60) for fast and easy detection of β-galactosidase expression.

Note: The β-galactosidase protein expressed from the Lenti6.3/TO/V5-GW/*lacZ* control lentiviral construct is fused to a V5 epitope and is approximately 121 kDa in size. If you are performing Western blot analysis, you may also use the Anti V5 Antibodies available from Invitrogen (see page 61 for ordering information) for detection. For more information, refer to www.invitrogen.com.

Generating a ViraPower™ T-REx™ Host Cell Line

Introduction

After you have performed the co-transduction procedure and established that your Lenti6.3/TO/V5-DEST construct can be inducibly expressed, you may wish to establish a stable cell line that constitutively expresses the Tet repressor and inducibly expresses your gene of interest. We recommend that you first create a stable cell line that expresses only the Tet repressor (i.e., ViraPower™ T-REx™ host cell line), and then use that cell line to create a second cell line which will inducibly express your gene of interest from the Lenti6.3/TO/V5-DEST lentiviral construct.

Several T-REx™ cell lines that stably express the Tet repressor are available from Invitrogen (see page 61 for ordering information). If you wish to assay for tetracycline-regulated expression of your gene of interest in 293, HeLa, CHO, or Jurkat cells, you may want to use one of the T-REx™ cell lines as the host for your Lenti6.3/TO/V5-DEST lentiviral construct. However, these cell lines do not contain the genetic elements WPRE and cPPT that enhance viral titer and expression (see **HiPerform™ Technology**, page 2). **Note that you can use these cell lines only for transient expression, because the Lenti6.3/TO/V5-DEST lentiviral expression construct also contains the Blasticidin selection marker, making stable cell line development not possible.**

Note: The T-REx™ cell lines stably express the Tet repressor from the pcDNA™6/TR expression plasmid. This plasmid is used to generate stable TetR-expressing cell lines in Invitrogen's T-REx™ System. Both pLenti3.3/TR and pcDNA™6/TR contain the same *TetR* gene. For more information about the T-REx™ cell lines or pcDNA™6/TR, refer to www.invitrogen.com or contact Technical Support (see page 62).

Materials Needed

- Titered Lenti3.3/TR lentiviral stock (store at –80°C until use)
- Mammalian cell line of choice
- Complete culture medium for your cell line
- 6 mg/mL Polybrene®, if desired
- Appropriately sized tissue culture plates for your application
- 10 mg/mL Blasticidin stock

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Lenti3.3/TR Transduction Procedure

Follow the procedure below for transducing the mammalian cell line of choice with the Lenti3.3/TR lentiviral construct and use Geneticin® selection to generate a ViraPower™ T-REx™ cell line. We recommend including a negative control (mock transduction) to help you evaluate your results.

1. Plate cells in complete growth media as appropriate.

Day 1:

2. On the day of transduction (Day 1), thaw the Lenti3.3/TR lentiviral stock and dilute (if necessary) the appropriate amount of virus (at a suitable MOI; recommended MOI = 10) into fresh complete medium. Keep the total volume of medium containing virus as low as possible to maximize transduction efficiency. **Do not vortex.**
3. Remove the culture medium from the cells. Mix the medium containing virus gently by pipetting and add to the cells.
4. Add Polybrene® (if desired) to a final concentration of 6 µg/mL. Swirl the plate gently to mix. Incubate at 37°C overnight.

Day 2:

5. The following day, remove the medium containing virus and replace with fresh, complete culture medium.

Day 3:

6. The following day, remove the medium and replace with fresh, complete medium containing the appropriate amount of Geneticin® to select for stably transduced cells.
7. Replace medium with fresh medium containing Geneticin® every 2–3 days until Geneticin®-resistant colonies can be identified (generally 10–12 days after selection).

   Note: Transducing cells with Lenti3.3/TR lentivirus at a high MOI results in most of the cells being Geneticin®-resistant. In this case, you may not be able to see distinct Geneticin®-resistant colonies when performing stable selection. You may also not see many non-transduced cells (i.e., dead cells).
8. Pick at least 10 Geneticin®-resistant colonies and expand each clone to assay for Tet repressor expression (see below). Alternatively, you may pool the heterogeneous population of Geneticin®-resistant cells and screen for Tet repressor expression.

Important

Integration of the lentivirus into the genome is random. Depending upon the influence of the surrounding genomic sequences at the integration site, you may see varying levels of Tet repressor expression from different Geneticin®-resistant clones.

When generating a stable cell line expressing the Tet repressor (i.e., ViraPower™ T-REx™ host cell line), select for clones that express the highest levels of Tet repressor to use as hosts for your inducible Lenti6.3/TO/V5-DEST expression construct. Those clones that express the highest levels of Tet repressor exhibit the most complete repression of basal transcription of your gene of interest.

Continued on next page

Detecting TetR Expression

To detect Tet repressor expression, we recommend performing Western blot analysis using an Anti-Tet repressor antibody (MoBiTec, Göttingen, Germany, Cat. no. TET01).

Maintaining the ViraPower™ T-REx™ Cell Line

After you have generated your ViraPower™ T-REx™ cell line and have verified that the cells express suitable levels of Tet repressor, we recommend that you:

- Maintain your ViraPower™ T-REx™ cell line in medium containing Geneticin®, and
- Freeze and store vials of early passage cells

Expressing the Gene of Interest

To express the gene of interest in a tetracycline-regulated manner, use the ViraPower™ T-Rex™ cell line as the host for your Lenti6.3/TO/V5-DEST lentiviral construct. After transducing the Lenti6.3/TO/V5-DEST lentivirus into the ViraPower™ T-REx™ cells, you have two options to express the gene of interest:

1. You may add tetracycline and assay for transient expression of the gene of interest

   or

2. You may use Blasticidin to select for a stable cell line, then add tetracycline to assay for expression of the gene of interest

Choose the option that best fits your needs.

Materials Needed

- Titered Lenti6.3/TO/V5-DEST lentiviral stock (store at –80°C until use)
- Your ViraPower™ T-REx™ host cell line cultured in medium containing Geneticin®
- Complete culture medium containing Geneticin®
- 6 mg/mL Polybrene®, if desired
- 10 mg/mL tetracycline (supplied with the kit, store **protected from light**)
- Appropriately sized tissue culture plates for your application
- 10 mg/mL Blasticidin stock (if selecting for stably transduced Lenti6.3/TO/V5-DEST cells)

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Lenti6.3/TO/V5-DEST Transduction Procedure

Follow the procedure below to transduce your ViraPower™ T-Rex™ cells with the Lenti6.3/TO/V5-DEST lentiviral construct and to use Blasticidin to generate a stable cell line. We recommend including a negative control (mock transduction) to help you evaluate your results.

1. Plate the ViraPower™ T-REx™ cells in complete growth media as appropriate for your application. If you plan to select for stably transduced cells, plate cells such that they will be 50–60% confluent on the day of transduction.

Day 1:

2. On the day of transduction (Day 1), thaw the Lenti6.3/TO/V5-DEST lentiviral stock and dilute (if necessary) the appropriate amount of virus (at a suitable MOI; recommended MOI = 1–5) into fresh complete medium containing Geneticin®. Keep the total volume of medium containing virus as low as possible to maximize transduction efficiency. **Do not vortex.**
3. Remove the culture medium from the cells. Mix the medium containing virus gently by pipetting and add to the cells.
4. Add Polybrene® (if desired) to a final concentration of 6 µg/mL. Swirl the plate gently to mix. Incubate at 37°C overnight.

Day 2:

5. The following day, remove the medium containing virus and replace with fresh, complete medium containing Geneticin®. Incubate at 37°C overnight.

Day 3:

6. The following day, perform one of the following:
   - **Transient expression experiments:** Remove the medium containing virus and replace with fresh, complete medium containing 1 µg/mL tetracycline. Incubate the cells at 37°C for 24–48 hours before assaying for expression of your recombinant protein. If you wish to assay the cells at a later time, continue to culture the cells or replate them into larger-sized tissue culture formats as necessary in medium containing tetracycline.
   - **Stable cell lines:** Trypsinize and replate cells into a larger-sized tissue culture format in fresh, complete medium containing Geneticin® and Blasticidin. Proceed to Step 7.
     Example: If transducing cells in a 6-well format, trypsinize and replate cells into a 10 cm tissue culture plate in medium containing Geneticin® and Blasticidin.

For stable cell lines only

7. Replace medium with fresh medium containing Geneticin® and Blasticidin every 2–3 days until you can identify Geneticin®- and Blasticidin-resistant colonies can be identified (generally 10–14 days after selection).
8. Pick at least 10 Geneticin®- and Blasticidin-resistant colonies and expand each clone. Alternatively, you may pool the heterogeneous population of Geneticin®- and Blasticidin-resistant cells.
9. Induce expression of the gene of interest by adding tetracycline to a final concentration of 1 µg/mL. Wait for the appropriate length of time (e.g., 24–48 hours) before assaying for your recombinant protein.

Troubleshooting

Generating the Lentiviral Stock

The table below lists some potential problems and possible solutions that may help you troubleshoot your co-transfection and titering experiments.

Problem	Reason	Solution
Low viral titer	Low transfection efficiency:	
	• Used poor quality expression construct plasmid DNA (i.e., plasmid DNA from a mini-prep)	• **Do not** use mini-prep plasmid DNA for transfection. Use the PureLink™ HiPure Plasmid Midiprep kit or CsCl gradient centrifugation to prepare plasmid DNA.
	• Unhealthy 293FT cells; cells exhibit low viability	• Use healthy 293FT cells under passage 16; do not overgrow. Culture cells for at least 3–4 passages before transfection.
	• Cells transfected in media containing antibiotics (i.e., Geneticin®)	• Although Geneticin® is required for stable maintenance of 293FT cells, **do not** add Geneticin® to media during transfection as this reduces transfection efficiency and causes cell death.
	• Plasmid DNA:transfection reagent ratio incorrect	• Use a DNA:Lipofectamine™ 2000 ratio ranging from 1:2 to 1:3 (in µg: µL).
	• Insufficient co-transfection	• Use more DNA/ Lipofectamine™ 2000 (keeping the ratios the same). For example, use 5 µg of lentiviral vector, 15 µg of packaging mix, and 60 µL of Lipofectamine™ 2000 for transfection.
	• 293FT cells plated too sparsely	• Plate cells such that they are 90–95% confluent at the time of transfection **or** use the Reverse Transfection protocol (i.e., add cells to media containing DNA-lipid complexes; see page 15).
	Transfected cells not cultured in media containing sodium pyruvate	One day after transfection, remove media containing DNA-lipid complexes and replace with media containing sodium pyruvate. Sodium pyruvate provides an extra energy source for the cells.
	Viral supernatant harvested too early	Viral supernatants can generally be collected 48–72 hours posttransfection. If many cells are still attached to the plate and look healthy at this point, wait an additional 24 hours before harvesting the viral supernatant. Harvest no later than 72 hours post-transfection.
	Viral supernatant too dilute	Concentrate your virus (Yee, 1999) .
	Viral supernatant frozen and thawed multiple times	Do **not** freeze/thaw viral supernatant more than 3 times.

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Generating the Lentiviral Stock, continued

Problem	Reason	Solution
Low viral titer, continued	Poor choice of titering cell line	Use HT1080 cells or another adherent cell line with the characteristics discussed on page 18.
	Gene of interest is toxic to cells	Do not generate constructs containing activated oncogenes or harmful genes.
	Gene of interest is large	Viral titers generally decrease as the size of the insert increases. Inserts larger than 5.6 kb are not recommended (see page 18). Concentrate the virus if titer is low (see page 26).
	Polybrene® not included during transduction	Transduce the lentiviral construct into cells in the presence of Polybrene®.
	Lipofectamine™ 2000 handled incorrectly	• Store at 4°C. **Do not freeze.** • Mix gently by inversion. **Do not vortex.**
No colonies obtained upon titering	Too much antibiotic used for selection	Determine the antibiotic sensitivity of your cell line by performing a kill curve experiment, and use the minimum concentration required to kill your untransduced cell line.
	Viral stocks stored incorrectly	Aliquot and store stocks at –80°C. Do not freeze/thaw more than 3 times.
	Polybrene® not included during transduction	Transduce the lentiviral construct into cells in the presence of Polybrene®.
Titer indeterminable; cells confluent	Too little antibiotic used for selection	Increase amount of antibiotic.
	Viral supernatant insufficiently diluted	Titer lentivirus using a wider range of 10-fold serial dilutions (e.g., 10^{-2} to 10^{-8}).
	Cells density too high for Geneticin® selection	Because Geneticin® is not very effective at high cell densities, it might be necessary to dilute your cells to select for stable transductants.

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Transducing Mammalian Cells

The table below lists some potential problems and possible solutions that may help you troubleshoot your transduction and expression experiment.

Problem	Reason	Solution
No expression of the gene of interest	Promoter silencing	Lentiviral constructs may integrate into a chromosomal region that silences the CMV promoter. Screen multiple antibiotic-resistant clones and select the one with the highest expression levels.
	Viral stocks stored incorrectly	Aliquot and store stocks at –80°C. Do not freeze/thaw more than 3 times.
Poor expression of the gene of interest	Low transduction efficiency: • Polybrene® not included during transduction • Non-dividing cell type used	• Transduce the lentiviral construct into cells in the presence of Polybrene®. • Transduce your lentiviral construct into cells using a higher MOI.
	MOI too low	Transduce your lentiviral construct into cells using a higher MOI.
	Too much antibiotic used for selection	Determine the antibiotic sensitivity of your cell line by performing a kill curve. Use the minimum antibiotic concentration required to kill your untransduced cell line.
	Cells harvested too soon after transduction	Do not harvest cells until at least 48–72 hours after transduction to allow expressed protein to accumulate in transduced cells.
	Gene of interest is toxic to cells	Generating constructs containing activated oncogenes or potentially harmful genes is not recommended.
Cytotoxic effects observed after transduction	Large volume of viral supernatant used for transduction	• Remove the "spent" media containing virus and replace with fresh, complete media. • Concentrate the virus (Yee, 1999).
	Your cells are sensitive to Polybrene®	Verify the sensitivity of your cells to Polybrene®. If cells are sensitive, omit the Polybrene® during transduction.
	Too much antibiotic used for selection	Determine the antibiotic sensitivity of your cell line by performing a kill curve. Use the minimum concentration of antibiotic required to kill your untransduced cell line.
	Gene of interest is toxic to cells	Try a different cell line.

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Transducing Mammalian Cells, continued

Low levels of Tet repressor expressed	Lenti3.3/TR construct integrated into an inactive region of the genome	Screen other Geneticin®-resistant colonies. Choose the clone that exhibits the highest level of Tet repressor expression for use as the host for your Lenti6.3/TO/V5-DEST construct.
	Transduced Lenti3.3/TR into a mammalian cell line in which the CMV promoter is down-regulated	Use another mammalian cell line for transduction.
Poor expression of the gene of interest	Cells harvested and assayed too soon after addition of tetracycline	• Culture cells for a longer period of time after addition of tetracycline before assaying for recombinant protein expression. Do not harvest cells until at least 24 hours after addition of tetracycline. • Placing cells under Geneticin® selection can improve gene knockdown results by killing untransduced cells.
	Lenti6.3/TO/V5-DEST lentiviral stock not titered	Titer the lentiviral stock using the procedure on page 21 before use.
	Lenti6.3/TO/V5-DEST lentiviral stock stored incorrectly	• Aliquot and store stocks at –80°C. • Do not freeze/thaw more than 3 times. • If stored for longer than 6 months, re-titer stock before use.
No tetracycline-regulated expression of the gene of interest **or** no gene expression	Did not transduce the Lenti6.3/TO/V5-DEST lentiviral construct into a Tet repressor-expressing cell line	• Generate a ViraPower™ T-Rex™ cell line first, then use this cell line as the host for the Lenti6.3/TO/V5-DEST virus. • Perform the co-transduction procedure (see pages 27–30). Make sure that the Lenti3.3/TR lentivirus is transduced into mammalian cells at least 24 hours before transduction of the Lenti6.3/TO/V5-DEST lentivirus.
	Forgot to add tetracycline	To induce expression of the gene of interest after transduction of Lenti6.3/TO/V5-DEST lentivirus, add tetracycline to a final concentration of 1 µg/mL. Wait for at least 24 hours before assaying for recombinant protein expression.
High basal level expression of the gene of interest	Did not transduce the Lenti6.3/TO/V5-DEST construct into Tet repressor-expressing cells	Use a ViraPower™ T-REx™ cell line as the host for your Lenti6.3/TO/V5-DEST lentiviral construct.

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Transducing Mammalian Cells, continued

High basal level expression of the gene of interest in transient co-transduction experiments	• Transduced Lenti3.3/TR viral construct at too low of an MOI when compared to the expression construct • Did not wait for a sufficient amount of time after transducing the Lenti3.3/TR viral construct before transducing the Lenti6.3/TO/V5-DEST viral construct	• Transduce the Lenti3.3/TR viral construct into mammalian cells at a higher MOI (e.g., MOI = 10) than the expression construct (e.g., MOI = 1–5). • Transduce mammalian cells with the Lenti6/TR construct, then wait for 24 hours before transducing cells with the Lenti6.3/TO/V5-DEST construct.

Appendix

Blasticidin

Description

Blasticidin S HCl is a nucleoside antibiotic isolated from *Streptomyces griseochromogenes* which inhibits protein synthesis in both prokaryotic and eukaryotic cells. Resistance is conferred by expression of either one of two Blasticidin S deaminase genes: *BSD* from *Aspergillus terreus* (Kimura *et al.*, 1994) or *bsr* from *Bacillus cereus* (Izumi *et al.*, 1991). These deaminases convert Blasticidin S to a non-toxic deaminohydroxy derivative (Izumi *et al.*, 1991).

Handling Blasticidin

Always wear gloves, mask, goggles, and a laboratory coat when handling Blasticidin. Weigh out Blasticidin and prepare solutions in a hood.

Preparing and Storing Stock Solutions

- Blasticidin is soluble in water and acetic acid.
- Prepare a stock solution of 5 to 10 mg/mL Blasticidin in sterile water and filter-sterilize the solution.
- Aliquot in small volumes suitable for one time use and freeze at –20°C for long-term storage or store at 4°C for short term storage.
- Aqueous stock solutions are stable for 1 week at 4°C and 6–8 weeks at –20°C.
- pH of the aqueous solution should not exceed 7.0 to prevent inactivation of Blasticidin.
- Do not subject stock solutions to freeze/thaw cycles (**do not store in a frost-free freezer**).
- Upon thawing, use what you need and discard the unused portion.
- Medium containing Blasticidin may be stored at 4°C for up to 2 weeks.

Determining Blasticidin Sensitivity

To select for stably transduced cells using Blasticidin, you must first determine the minimum concentration of Blasticidin required to kill your untransduced mammalian cell line (i.e., perform a kill curve experiment). Typically, concentrations ranging from 2–10 μg/mL Blasticidin are sufficient to kill most untransduced mammalian cell lines. We recommend that you test a range of concentrations (see protocol below) to ensure that you determine the minimum concentration necessary for your cell line.

1. Plate cells at approximately 25% confluence. Prepare a set of 7 plates. Allow the cells to adhere overnight.
2. The next day, substitute culture medium with medium containing varying concentrations of Blasticidin, as appropriate.
3. Replenish the selective media every 3–4 days and observe the percentage of surviving cells.
4. Determine the appropriate concentration of Blasticidin that kills the cells within 10–14 days after addition of antibiotic.

Geneticin®

Geneticin® (G-418)

The pLenti3.3/TR vector contains the neomycin resistance gene which confers resistance to the antibiotic Geneticin® (also known as G-418 sulfate). Geneticin® blocks protein synthesis in mammalian cells by interfering with ribosomal function. It is an aminoglycoside, similar in structure to neomycin, gentamycin, and kanamycin. Expression in mammalian cells of the bacterial gene (APH), derived from Tn*5*, results in detoxification of Geneticin® (Southern & Berg, 1982).

Note: Geneticin® is also available separately from Invitrogen (see page 60 for ordering information).

Geneticin® is harmful. May cause sensitization by skin contact. Irritating to eyes and skin. In case of contact with eyes, rinse immediately with plenty of water and seek medical advice. Avoid contact with skin and eyes. Wear suitable protective clothing and gloves when handling Geneticin® and Geneticin®-containing solutions.

Preparing and Storing Geneticin®

Follow the instructions provided with Geneticin® to prepare your working stock solution. Geneticin® in powder form should be stored at room temperature and at 4°C as a solution. The stability of Geneticin® is guaranteed for two years, if stored properly.

Determining Geneticin® Sensitivity

The amount of Geneticin® required in culture media to select for resistant cells depends on a number of factors, including cell type. Although the development work with this kit utilized 293FT cells and 500 µg/mL Geneticin®, we recommend that you re-evaluate the optimal Geneticin® concentration whenever experimental conditions are altered (including use of Geneticin® from a different lot). Note that Geneticin® in powder form has only 75% of the potency of Geneticin® available in liquid form.

1. Plate or split a confluent plate so the cells will be approximately 25% confluent. Prepare a set of 7 plates. Allow cells to adhere overnight.
2. The next day, substitute culture medium with medium containing varying concentrations of Geneticin® (0, 50, 100, 250, 500, 750, and 1000 µg/mL Geneticin®).
3. Replenish the selective media every 3–4 days, and observe the percentage of surviving cells.
4. Note the percentage of surviving cells at regular intervals to determine the appropriate concentration of Geneticin® that kills the cells within 1–2 weeks after addition of Geneticin®.

Note: Cells will divide once or twice in the presence of lethal doses of Geneticin®, because the effects of the drug take several days to become apparent. Complete selection can take up to two weeks of growth in selective medium.

Change in 293FT Morphology

Introduction

During lentivirus production, expression of the VSV G glycoprotein causes transfected 293FT cells to fuse, resulting in the appearance of large, multinucleated cells known as syncytia, while untransfected 293FT cells leave empty spaces on the surface of the culture dish and pile up at other spots of the dish.

The following series of images show 293FT cells before and at 6, 24, and 48 hours after transfection with the Vivid Colors™ pLenti6.3/V5-GW/EmGFP expression control vector, which constitutively expresses the Emerald Green Fluorescent Protein (EmGFP).

Note: The Vivid Colors™ pLenti6.3/V5-GW/EmGFP expression control vector is available separately from Invitrogen; see page 60 for ordering information.

Untransfected 293FT Cells

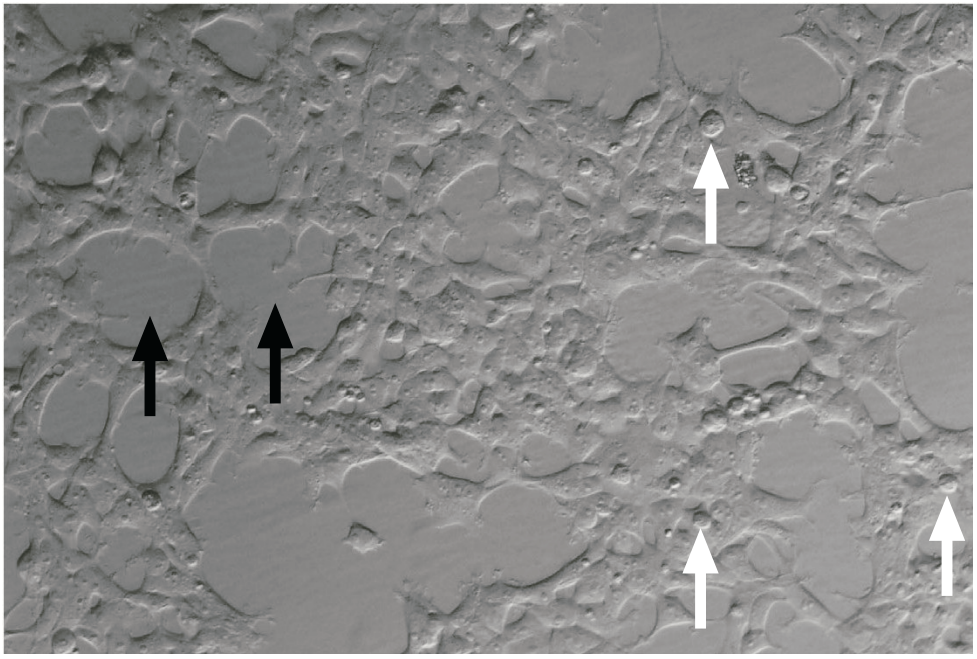


Figure 1. Bright field image of 293FT culture one day before transfection. The cells have been cultured in complete growth medium for 10 days. White arrows point to individual 293FT cells, and black arrows point to empty spaces on the surface of the culture dish.

Continued on next page

293FT Cells
6 Hours
Post-Transfection

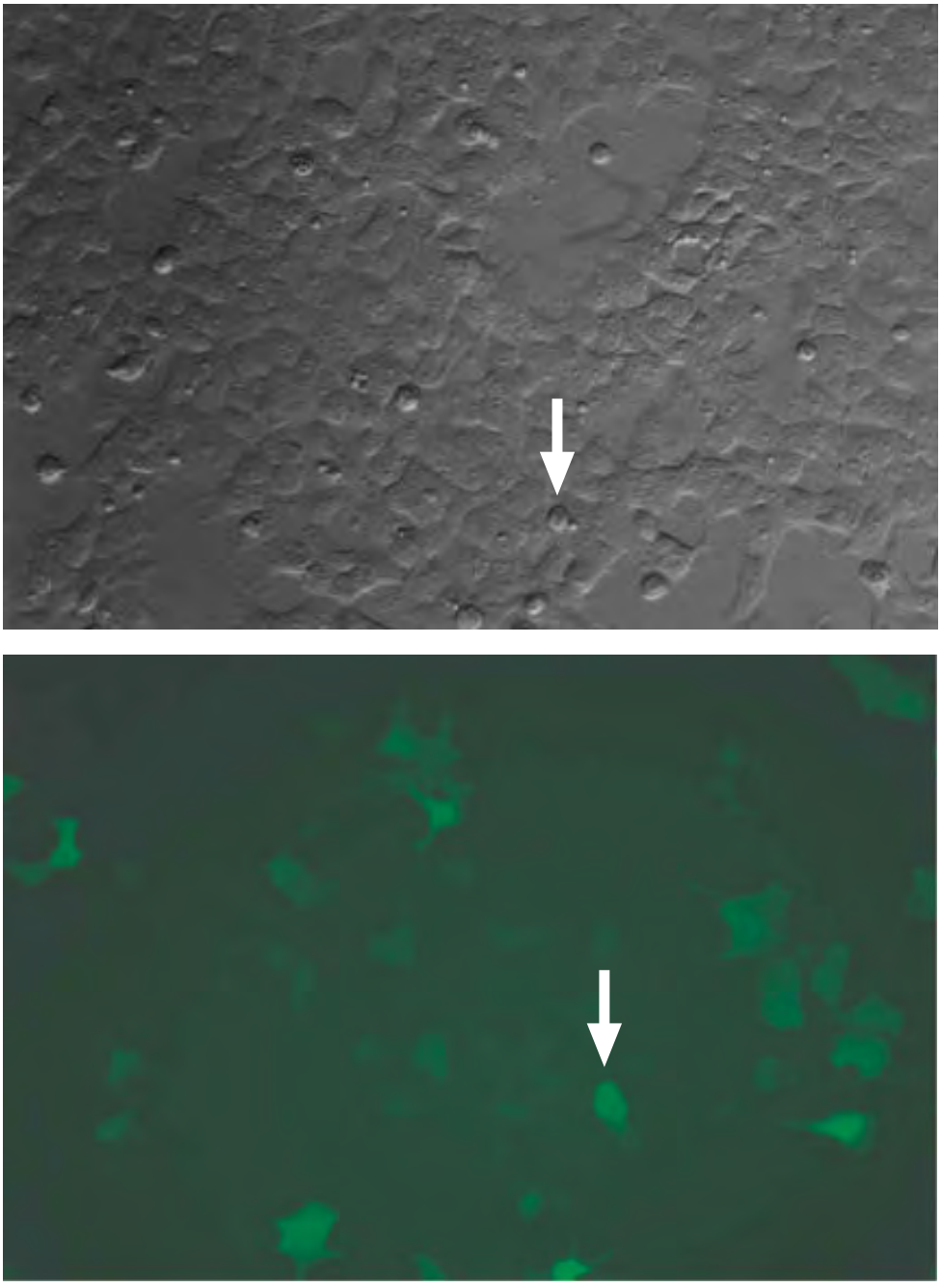


Figure 2. Bright field (top panel) and fluorescent (bottom panel) images of 293FT cells 6 hours after transfection with the pLenti6.3/V5-GW/EmGFP expression control vector. White arrows point to individual 293FT cells. EmGFP expression is apparent, but the cells do not yet show signs of lentivirus production.

Continued on next page

293FT Cells 24 Hours Post-Transfection

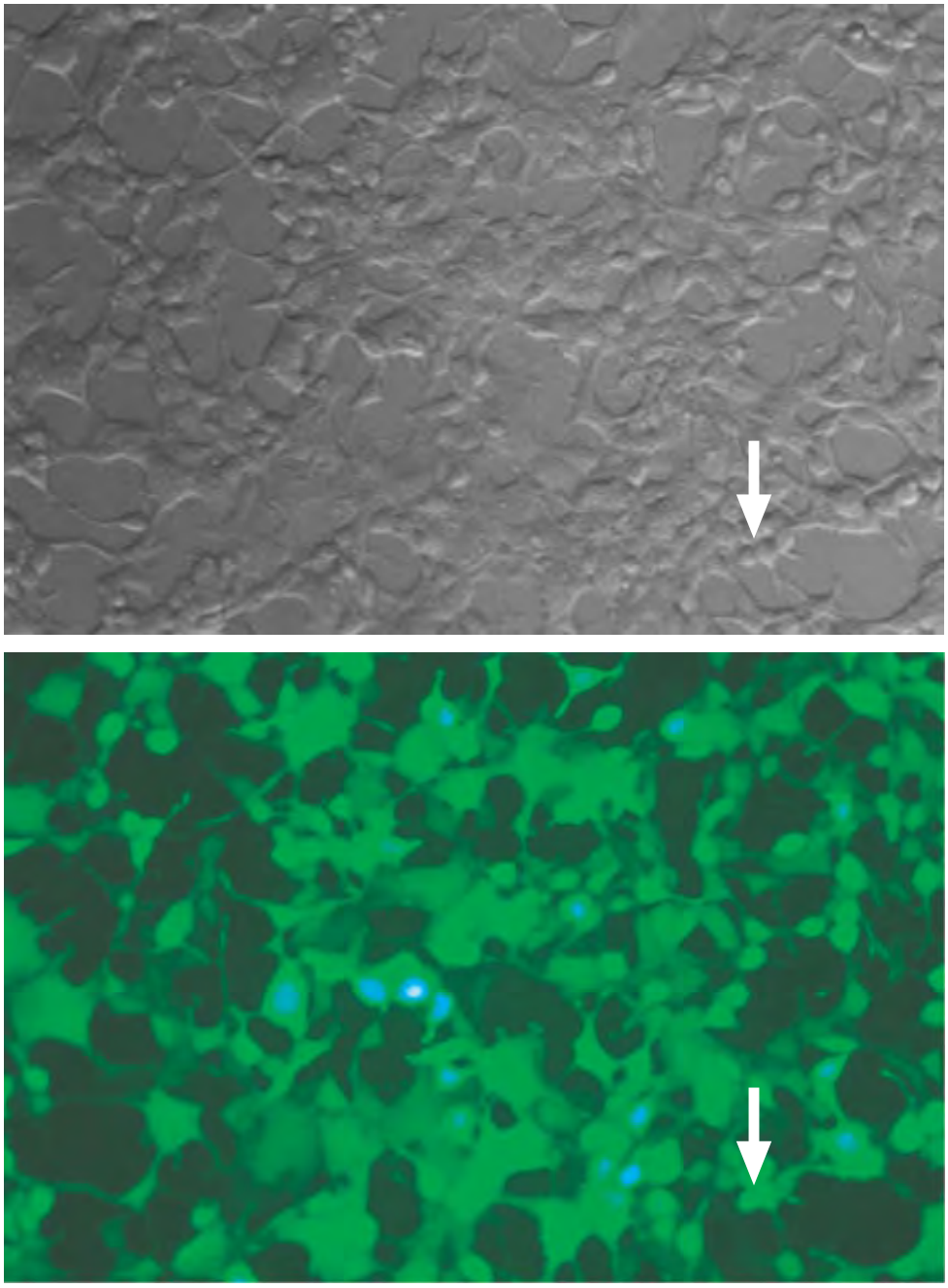


Figure 3. Bright field (top panel) and fluorescent (bottom panel) images of 293FT cells 24 hours after transfection with the pLenti6.3/V5-GW/EmGFP expression control vector. White arrow points to 293FT cells fusing together and becoming larger. EmGFP expression is increased, and the cells show weak signs of lentivirus production.

Continued on next page

293FT Cells
48 Hours
Post-Transfection

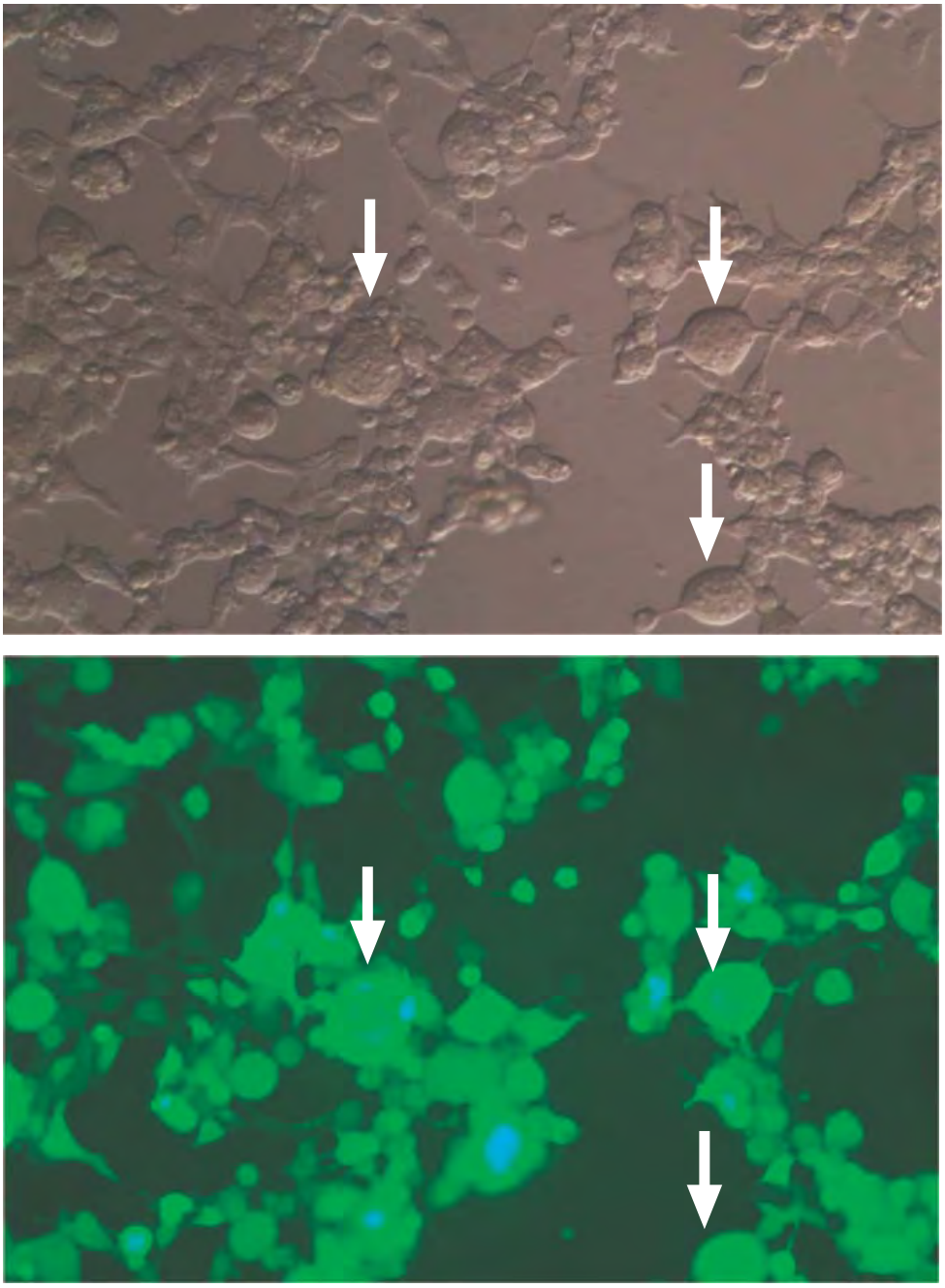
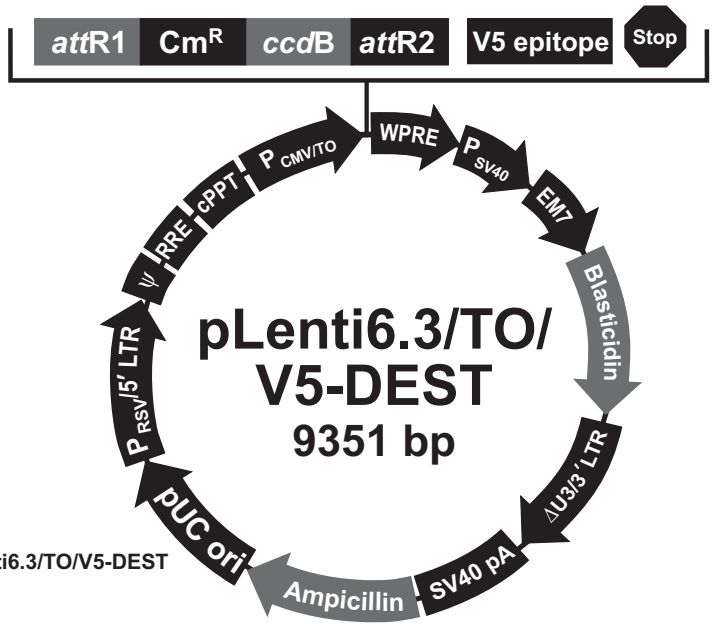


Figure 4. Bright field (top panel) and fluorescent (bottom panel) images of 293FT cells 48 hours after transfection with the pLenti6.3/V5-GW/EmGFP expression control vector. White arrow points to 293FT cells that have fused together. Note the "balloon-like" appearance of these multinucleated cells, which show signs of increased lentivirus production.

Map and Features of pLenti6.3/TO/V5-DEST

pLenti6.3/TO/V5-DEST Map

The map below shows the elements of pLenti6.3/TO/V5-DEST. DNA from the entry clone replaces the region between bases 2,539 and 4,222. **The complete sequence for pLenti6.3/TO/V5-DEST is available at www.invitrogen.com or by contacting Technical Support (see page 62).**



Comments for pLenti6.3/TO/V5-DEST
9351 nucleotides

RSV/5′ LTR hybrid promoter: bases 1-410
- RSV promoter: bases 1-229
- HIV-1 5′ LTR: bases 230-410

5′ splice donor: base 520
HIV-1 psi (ψ) packaging signal: bases 521-565
HIV-1 Rev response element (RRE): bases 1075-1308
3′ splice acceptor: base 1656
3′ splice acceptor: base 1684
cPPT: bases 1801-1923
CMV/TO promoter: bases 1937-2491
- CMV promoter: bases 1937-2436
- TATA box: bases 2436-2442
- Tetracycline operator (2X $TetO_2$) sequences: bases 2452-2491

*att*R1 site: bases 2532-2656
Chloramphenicol resistance gene (Cm^R): bases 2765-3424
*ccd*B gene: bases 3766-4071
*att*R2 site: bases 4112-4236
V5 epitope: bases 4289-4330
WPRE: bases 4349-4946
SV40 promoter: bases 4957-5265
EM7 promoter: bases 5320-5386
Blasticidin resistance gene: bases 5387-5785
ΔU3/3′ LTR: bases 5871-6105
- ΔU3: bases 5871-5924
- 3′ LTR: bases 5925-6105

SV40 polyadenylation signal: bases 6177-6308
bla promoter: bases 7167-7265
Ampicillin (*bla*) resistance gene: bases 7266-8126
pUC origin: bases 8271-8944

Continued on next page

Map and Features of pLenti6.3/TO/V5-DEST, Continued

Features of pLenti6.3/TO/V5-DEST

pLenti6.3/TO/V5-DEST (9,351 bp) contains the following elements. Features have been functionally tested.

Feature	Benefit
Rous Sarcoma Virus (RSV) enhancer/promoter	Allows Tat-independent production of viral mRNA (Dull *et al.*, 1998).
HIV-1 truncated 5′ LTR	Allows viral packaging and reverse transcription of the viral mRNA (Luciw, 1996).
5′ splice donor and 3′ acceptors	Enhances the biosafety of the vector by facilitating removal of the Ψ packaging sequence and RRE such that expression of the gene of interest in the transduced host cell is no longer Rev-dependent (Dull *et al.*, 1998).
HIV-1 psi (ψ) packaging signal	Allows viral packaging (Luciw, 1996).
HIV-1 Rev response element (RRE)	Allows Rev-dependent nuclear export of unspliced viral mRNA (Kjems *et al.*, 1991; Malim *et al.*, 1989).
Polypurine Tract from HIV (cPPT)	Provides for increased viral titer (Park, 2001).
CMV/TO promoter	Hybrid promoter consisting of the human cytomegalovirus promoter (Andersson *et al.*, 1989; Boshart *et al.*, 1985; Nelson *et al.*, 1987) and two tandem tetracycline operator (O_2) sequences for high-level, inducible expression of the gene of interest. The tetracycline operator sequences serve as binding sites for Tet repressor homodimers (Hillen & Berens, 1994).
*att*R1 and *att*R2 sites	Bacteriophage λ-derived DNA recombination sequences that permit recombinational cloning of the gene of interest from a Gateway® entry clone (Landy, 1989).
Chloramphenicol resistance gene (Cm^R)	Allows counterselection of the plasmid.
*ccd*B gene	Allows negative selection of the plasmid.
V5 epitope	Allows detection of the recombinant fusion protein using the Anti-V5 Antibodies (Southern *et al.*, 1991).
Woodchuck Posttranscriptional Regulatory Element (WPRE)	Provides for increased transgene expression (Zufferey *et al.*, 1998)
SV40 early promoter and origin	Allows high-level expression of the selection marker and episomal replication in cells expressing the SV40 large T antigen.
EM7 promoter	Synthetic prokaryotic promoter for expression of the selection marker in *E. coli.*
Blasticidin (*bsd*) resistance gene	Allows selection of stably transduced mammalian cell lines (Kimura *et al.*, 1994).

Continued on next page

Map and Features of pLenti6.3/TO/V5-DEST, Continued

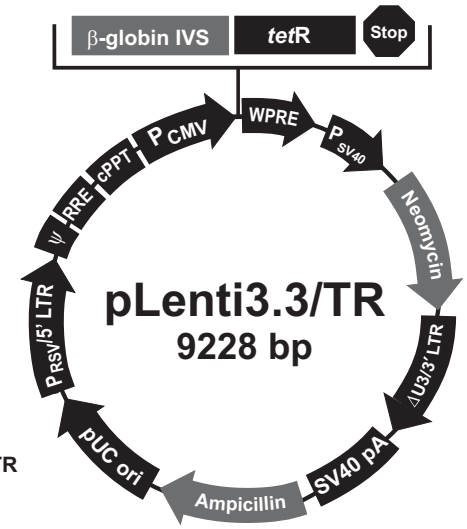
Features of pLenti6.3/TO/V5-DEST, continued

ΔU3/HIV-1 truncated 3′ LTR	Allows viral packaging but self-inactivates the 5′ LTR for biosafety purposes (Dull *et al.,* 1998). The element also contains a polyadenylation signal for transcription termination and polyadenylation of mRNA in transduced cells.
SV40 polyadenylation signal	Allows transcription termination and polyadenylation of mRNA.
bla promoter	Allows expression of the ampicillin resistance gene.
Ampicillin resistance gene (β-lactamase)	Allows selection of the plasmid in *E. coli*.
pUC origin	Allows high-copy replication and maintenance in *E. coli*.

Map and Features of pLenti3.3/TR

pLenti3.3/TR Map

The map below shows the elements of pLenti3.3/TR. **The complete sequence for pLenti3.3/TR is available at www.invitrogen.com or by contacting Technical Support (see page 62).**



Comments for pLenti3.3/TR
9228 nucleotides

RSV/5' LTR hybrid promoter: bases 1-410
RSV promoter: bases 1-229
HIV-1 5' LTR: bases 230-410
5' splice donor: base 520
HIV-1 psi (ψ) packaging signal: bases 521-565
HIV-1 Rev response element (RRE): bases 1075-1308
3' splice acceptor: base 1656
3' splice acceptor: base 1684
cPPT: bases 1801-1923
CMV promoter: bases 1935-2519
Rabbit β-globin intron II (IVS): bases 2567-3139
*tet*R gene: bases 3223-3870
WPRE: bases 3892-4489
SV40 promoter: bases 4500-4808
Neomycin resistance gene: bases 4883-5677
ΔU3/3' LTR: bases 5748-5982
ΔU3: bases 5748-5801
3′ LTR: bases 5802-5982
SV40 polyadenylation signal: bases 6054-6185
bla promoter: bases 7044-7142
Ampicillin (*bla*) resistance gene: bases 7143-8003
pUC origin: bases 8148-8821

TetR Gene

The *TetR* gene in pLenti3.3/TR was originally isolated from the Tn*10* transposon which confers resistance to tetracycline in *E. coli* and other enteric bacteria (Postle *et al.*, 1984). The *TetR* gene from Tn*10* encodes a class B Tet repressor and is often referred to as *TetR(B)* in the literature (Hillen & Berens, 1994).

The *TetR* gene encodes a repressor protein of 207 amino acids with a calculated molecular weight of 23 kDa. For more information about the Tet repressor and its interaction with the Tet operator, refer to the review by Hillen and Berens, 1994.

Continued on next page

Map and Features of pLenti3.3/TR, Continued

Features of pLenti3.3/TR

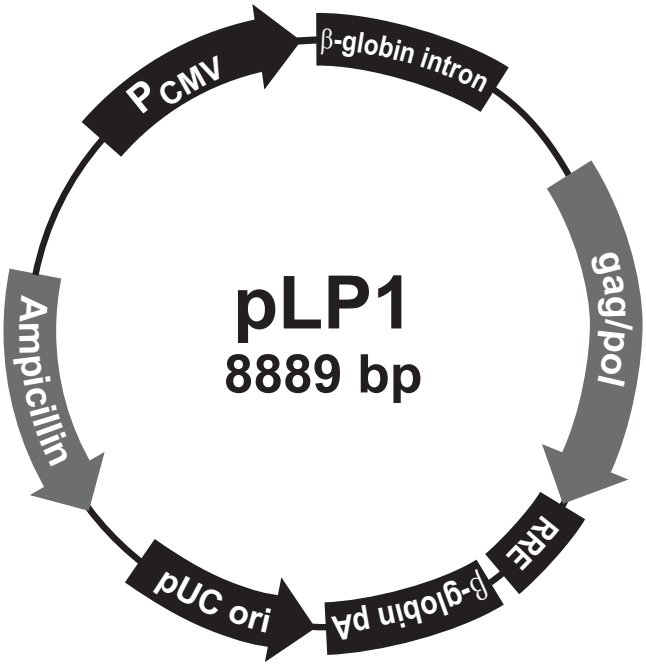
pLenti3.3/TR (9,228 bp) contains the following elements. Features have been functionally tested.

Feature	Benefit
Rous Sarcoma Virus (RSV) enhancer/promoter	Allows Tat-independent production of viral mRNA (Dull *et al.*, 1998).
HIV-1 truncated 5′ LTR	Allows viral packaging and reverse transcription of the viral mRNA (Luciw, 1996).
5′ splice donor and 3′ acceptors	Enhances the biosafety of the vector by facilitating removal of the Ψ packaging sequence and RRE such that expression of the gene of interest in the transduced host cell is no longer Rev-dependent (Dull *et al.*, 1998).
HIV-1 psi (ψ) packaging signal	Allows viral packaging (Luciw, 1996).
HIV-1 Rev response element (RRE)	Allows Rev-dependent nuclear export of unspliced viral mRNA (Kjems *et al.*, 1991; Malim *et al.*, 1989).
Polypurine Tract from HIV (cPPT)	Provides for increased viral titer (Park, 2001)
CMV promoter	Allows high-level, constitutive expression of the Tet repressor in mammalian cells (Andersson *et al.*, 1989; Boshart *et al.*, 1985; Nelson *et al.*, 1987).
Rabbit β-globin intron II (IVS)	Enhances expression of the *TetR* gene in mammalian cells (van Ooyen *et al.*, 1979).
*Tet*R gene	Encodes the Tet repressor that binds to tet operator sequences to repress transcription of the gene of interest in the absence of tetracycline (Postle *et al.*, 1984; Yao *et al.*, 1998).
Woodchuck Posttranscriptional Regulatory Element (WPRE)	Provides for increased transgene expression (Zufferey *et al.*, 1998)
SV40 early promoter and origin	Allows high-level expression of the selection marker and episomal replication in cells expressing the SV40 large T antigen.
EM7 promoter	Synthetic prokaryotic promoter for expression of the selection marker in *E. coli.*
Neomycin resistance gene	Allows selection of stably transduced mammalian cell lines.
ΔU3/HIV-1 truncated 3′ LTR	Allows viral packaging but self-inactivates the 5′ LTR for biosafety purposes (Dull *et al.*, 1998). The element also contains a polyadenylation signal for transcription termination and polyadenylation of mRNA in transduced cells.
SV40 polyadenylation signal	Allows transcription termination and polyadenylation of mRNA.
bla promoter	Allows expression of the ampicillin resistance gene.
Ampicillin resistance gene (β-lactamase)	Allows selection of the plasmid in *E. coli*.
pUC origin	Allows high-copy replication and maintenance in *E. coli*.

Map and Features of pLP1

pLP1 Map

The figure below shows the features of the pLP1 vector. Note that the *gag* and *pol* genes are initially expressed as a gag/pol fusion protein, which is then self-cleaved by the viral protease into individual Gag and Pol polyproteins. **The vector sequence of pLP1 is available for downloading from our website at www.invitrogen.com or by contacting Technical Support (see page 62).**



Comments for pLP1
8889 nucleotides

CMV promoter: bases 1-747
    TATA box: bases 648-651
Human β-globin intron: bases 880-1320
HIV-1 gag/pol sequences: bases 1355-5661
    gag coding sequence: bases 1355-2857
    gag/pol frameshift: base 2650
    pol coding sequence: bases 2650-5661
HIV-1 Rev response element (RRE): bases 5686-5919
Human β-globin polyadenylation signal: bases 6072-6837
pUC origin: bases 6995-7668 (C)
Ampicillin (*bla*) resistance gene: bases 7813-8673 (C)
bla promoter: bases 8674-8772 (C)

C=complementary strand

Continued on next page

Map and Features of pLP1, Continued

Features of pLP1

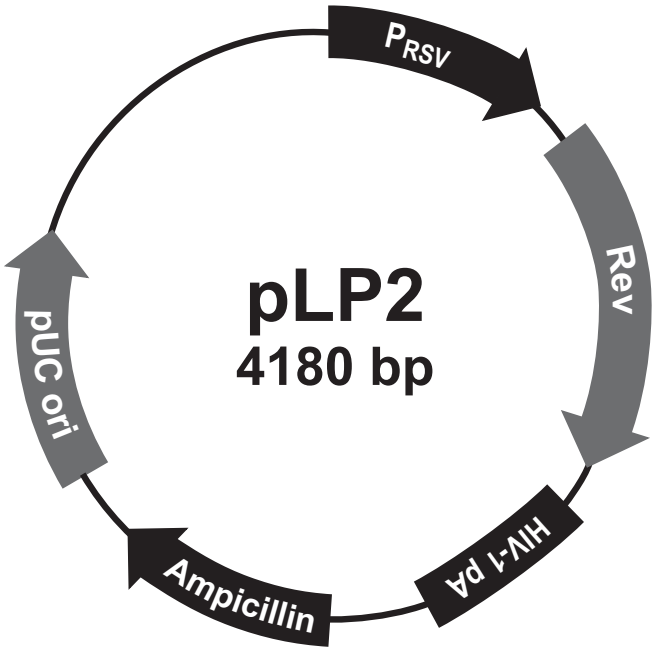
pLP1 (8,889 bp) contains the following elements. Features have been functionally tested.

Feature	Benefit
Human cytomegalovirus (CMV) promoter	Permits high-level expression of the HIV-1 *gag* and *pol* genes in mammalian cells (Andersson *et al.*, 1989; Boshart *et al.*, 1985; Nelson *et al.*, 1987).
Human β-globin intron	Enhances expression of the *gag* and *pol* genes in mammalian cells.
HIV-1 *gag* coding sequence	Encodes the viral core proteins required for forming the structure of the lentivirus (Luciw, 1996).
HIV-1 *pol* coding sequence	Encodes the viral replication enzymes required for replication and integration of the lentivirus (Luciw, 1996).
HIV-1 Rev response element (RRE)	Permits Rev-dependent expression of the *gag* and *pol* genes
Human β-globin polyadenylation signal	Allows efficient transcription termination and polyadenylation of mRNA.
pUC origin of replication (*ori*)	Permits high-copy replication and maintenance in *E. coli*.
Ampicillin (*bla*) resistance gene	Allows selection of the plasmid in *E. coli*.

Map and Features of pLP2

pLP2 Map

The figure below shows the features of the pLP2 vector. **The vector sequence of pLP2 is available for downloading from www.invitrogen.com or by contacting Technical Support (see page 62).**



Comments for pLP2
4180 nucleotides

RSV enhancer/promoter: bases 1-271
TATA box: bases 200-207
Transcription initiation site: base 229
RSV UTR: bases 230-271
HIV-1 Rev ORF: bases 391-741
HIV-1 LTR polyadenylation signal: bases 850-971
bla promoter: bases 1916-2014
Ampicillin (*bla*) resistance gene: bases 2015-2875
pUC origin: bases 3020-3693

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Map and Features of pLP2, Continued

Features of pLP2

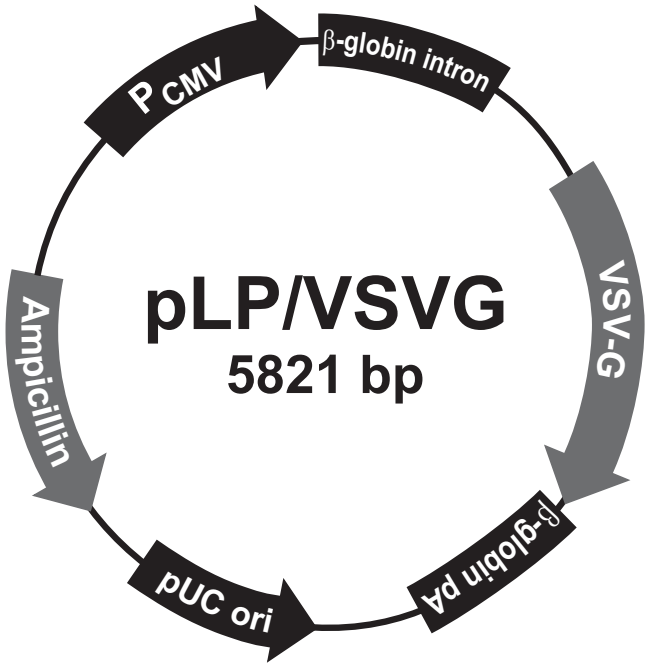
pLP2 (4,180 bp) contains the following elements. Features have been functionally tested.

Feature	Benefit
RSV enhancer/promoter	Permits high-level expression of the *rev* gene (Gorman *et al.*, 1982).
HIV-1 Rev ORF	Encodes the Rev protein that interacts with the RRE on pLP1 to induce Gag and Pol expression, and on the pLenti6.3/TO/V5-DEST or pLenti3.3/TR expression vector to promote the nuclear export of the unspliced viral RNA for packaging into viral particles.
HIV-1 LTR polyadenylation signal	Allows efficient transcription termination and polyadenylation of mRNA.
Ampicillin (*bla*) resistance gene	Allows selection of the plasmid in *E. coli*.
pUC origin of replication (*ori*)	Permits high-copy replication and maintenance in *E. coli*.

Map and Features of pLP/VSVG

pLP/VSVG Map

The figure below shows the features of the pLP/VSVG vector. **The vector sequence of pLP/VSVG is available for downloading from our website at www.invitrogen.com or by contacting Technical Support (see page 62).**



Comments for pLP/VSVG
5821 nucleotides

CMV promoter: bases 1-747
TATA box: bases 648-651
Human β-globin intron: bases 880-1320
VSV G glycoprotein (VSV-G): bases 1346-2881
Human β-globin polyadenylation signal: bases 3004-3769
pUC origin: bases 3927-4600 (C)
Ampicillin (*bla*) resistance gene: bases 4745-5605 (C)
bla promoter: bases 5606-5704 (C)

C=complementary strand

Continued on next page

Map and Features of pLP/VSVG, Continued

Features of pLP/VSVG

pLP/VSVG (5,821 bp) contains the following elements. Features have been functionally tested.

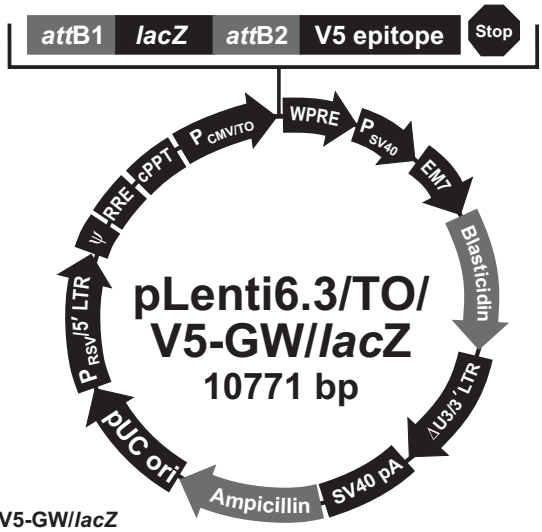
Feature	Benefit
Human CMV promoter	Permits high-level expression of the VSV-G gene in mammalian cells (Andersson *et al.*, 1989; Boshart *et al.*, 1985; Nelson *et al.*, 1987).
Human β-globin intron	Enhances expression of the VSV-G gene in mammalian cells.
VSV G glycoprotein (VSV-G)	Encodes the envelope G glycoprotein from Vesicular Stomatitis Virus to allow production of a pseudotyped retrovirus with a broad host range (Burns *et al.*, 1993; Emi *et al.*, 1991; Yee *et al.*, 1994).
Human β-globin polyadenylation signal	Allows efficient transcription termination and polyadenylation of mRNA.
pUC origin of replication (*ori*)	Permits high-copy replication and maintenance in *E. coli*.
Ampicillin (*bla*) resistance gene	Allows selection of the plasmid in *E. coli*.

Map of pLenti6.3/TO/V5-GW/*lacZ*

pLenti6.3/TO/V5-GW/*lacZ* Map

pLenti6.3/TO/V5-GW/*lacZ* is a 10,771 bp control vector expressing β-galactosidase, and was generated using the Gateway® LR recombination reaction between an entry clone containing the *lacZ* gene and pLenti6.3/TO/V5-DEST. β-galactosidase is expressed as a C-terminal V5 fusion protein with a molecular weight of approximately 121 kDa.

The map below shows the elements of pLenti6.3/TO/V5-GW/*lacZ*. **The complete sequence for pLenti6.3/TO/V5-GW/*lacZ* is available at www.invitrogen.com or by contacting Technical Support (see page 62).**



Comments for pLenti6.3/TO/V5-GW/*lacZ*
10771 nucleotides

RSV enhancer/promoter: bases 1-229
HIV-1 5′ LTR: bases 230-410
5′ splice donor: base 520
HIV-1 psi (ψ) packaging signal: bases 521-565
HIV-1 Rev response element (RRE): bases 1075-1308
3′ splice acceptor: base 1656
3′ splice acceptor: base 1684
cPPT: bases 1801-1923
CMV/TO promoter: bases 1937-2442
  CMV promoter: bases 1937-2435
  TATA box: bases 2436-2442
Tetracycline operator (2X $TetO_2$) sequences: bases 2452-2491
*att*B1 site: bases 2533-2553
lacZ ORF: bases 2559-5630
*att*B2 site: bases 5644-5656
V5 epitope: bases 5709-5750
WPRE: bases 5769-6366
SV40 early promoter and origin: bases 6377-6685
EM7 promoter: bases 6740-6806
Blasticidin resistance gene: bases 6807-7205
ΔU3/HIV-1 3′ LTR: bases 7291-7525
  ΔU3: bases 7291-7344
  Truncated HIV-1 3′ LTR: bases 7345-7525
SV40 polyadenylation signal: bases 7597-7728
bla promoter: bases 8587-8685
Ampicillin (*bla*) resistance gene: bases 8686-9546
pUC origin: bases 9691-10364

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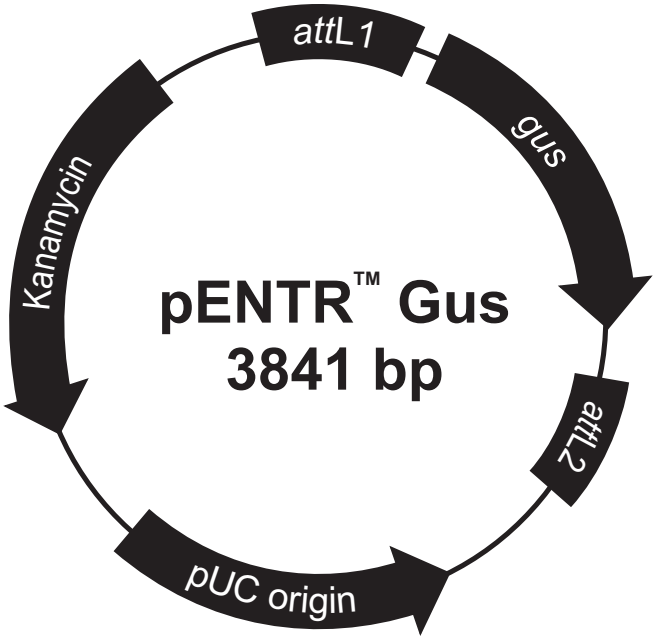
Map of pENTR™ Gus

pENTR™ Gus Map

pENTR™ Gus is a 3,841 bp entry clone containing the *Arabidopsis thaliana* gene for β-glucuronidase (*gus*) (Kertbundit *et al.*, 1991). The map below shows the elements of pENTR™ Gus. **The complete sequence for pENTR™ Gus is available at www.invitrogen.com or by contacting Technical Support (see page 62).**



Comments for pENTR™ Gus
3841 nucleotides

*att*L1: bases 99-198 (complementary strand)
gus gene: bases 228-2039
*att*L2: bases 2041-2140
pUC origin: bases 2200-2873 (C)
Kanamycin resistance gene: bases 2990-3805 (C)

C = complementary strand

Accessory Products

Additional Products

Many of the reagents supplied in the ViraPower™ HiPerform™ T-REx™ Gateway® Expression System and other products suitable for use with the kits are available separately from Invitrogen. Ordering information for these reagents is provided below. For more information, refer to www.invitrogen.com or contact Technical Support (page 62).

Item	Quantity	Cat. no.
pLenti6.3/V5 TOPO® TA Cloning Kit	20 reactions	K5315-20
pLenti7.3/V5-TOPO® TA Cloning Kit	20 reactions	K5325-20
ViraPower™ Packaging Mix	60 reactions	K4975-00
LR Clonase™ II Plus Enzyme Mix	20 reactions	12538-120
Vivid Colors™ pLenti6.3/V5-GW/EmGFP Expression Control Vector	20 µg	V370-06
PureLink™ HiPure Plasmid Midiprep Kit	25 reactions	K2100-04
	50 reactions	K2100-05
One Shot® Stbl3™ Chemically Competent *E. coli*	20 × 50 µL	C7373-03
293FT Cell Line	3×10^6 cells, frozen	R700-07
Lipofectamine™ 2000	0.75 mL	11668-027
	1.5 mL	11668-019
Opti-MEM® I Reduced Serum Medium	100 mL	31985-062
	500 mL	31985-070
Dulbecco's Modified Eagle Medium (D-MEM)	500 mL	11965-092
	1000 mL	11965-084
MEM Sodium Pyruvate Solution, 100 mM (100X), liquid	100 mL	11360-070
Blasticidin S HCl	50 mg	R210-01
Geneticin®, liquid	20 mL	10131-035
	100 mL	10131-027
Fetal Bovine Serum (FBS), Certified	500 mL	16000-044
Phosphate-Buffered Saline (PBS), pH 7.4	500 mL	10010-023
	1 L	10010-031
β-Gal Assay Kit	50 mL	K1455-01

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Detection of Recombinant Protein

If you have cloned your gene of interest in frame with the V5 epitope and your gene of interest does not contain a stop codon, you may detect expression of your recombinant fusion protein using an antibody to the V5 epitope (see table below). Horseradish peroxidase (HRP) or alkaline phosphatase (AP)-conjugated antibodies allow one-step detection using chemiluminescent or colorimetric detection methods. A fluorescein isothiocyanate (FITC)-conjugated antibody allows one-step detection in immunofluorescence experiments. The amount of antibody supplied is sufficient for 25 western blots or 25 immunostaining reactions, as appropriate.

Product	Quantity	Cat. no.
Anti-V5 Antibody	50 µL	R960-25
Anti-V5-HRP Antibody	50 µL	R961-25
Anti-V5-AP Antibody	125 µL	R962-25
Anti-V5-FITC Antibody	50 µL	R963-25

T-REx™ Cell Lines

Invitrogen has a number of cell lines available that stably express the Tet repressor from pcDNA™6/TR (TetR expressing plasmid from the T-REx™ System). The cell lines should be maintained in medium containing Blasticidin. **Note that you can use these cell lines only for transient expression, because the Lenti6.3/TO/V5-DEST lentiviral expression construct also contains the Blasticidin selection marker, making stable cell line development not possible.** For more information about pcDNA™6/TR and the T-REx™ system, refer to our website at www.invitrogen.com or contact Technical Support (page 62).

Item	Quantity	Cat. no.
T-REx™-293 Cell Line	3×10^6 cells, frozen	R710-07
T-REx™-HeLa Cell Line	3×10^6 cells, frozen	R714-07
T-REx™-CHO Cell Line	3×10^6 cells, frozen	R718-07
T-REx™-Jurkat Cell Line	3×10^6 cells, frozen	R722-07

Technical Support

Web Resources

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E-mail: eurotech@invitrogen.com

MSDS

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Introduction

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Purchaser Notification, Continued

Information for European Customers

The 293FT cell line is genetically modified and carries the pUC-derived plasmid, pCMVSPORT6TAg.neo. As a condition of sale, this product must be in accordance with all applicable local legislation and guidelines including EC Directive 90/219/EEC on the contained use of genetically modified organisms.

Gateway® Clone Distribution Policy

Introduction

The information supplied in this section is intended to provide clarity concerning Invitrogen's policy for the use and distribution of cloned nucleic acid fragments, including open reading frames, created using Invitrogen's commercially available Gateway® Technology.

Gateway® Entry Clones

Invitrogen understands that Gateway® entry clones, containing *att*L1 and *att*L2 sites, may be generated by academic and government researchers for the purpose of scientific research. Invitrogen agrees that such clones may be distributed for scientific research by non-profit organizations and by for-profit organizations without royalty payment to Invitrogen.

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